

Promoting Stress Resistance and Lifespan by
Modulators of Mitochondrial Metabolism

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Table of contents

Table of contents	I
Summary	V
Zusammenfassung	VI
List of abbreviations	VII
List of figures	IX
List of tables	XII
1. Introduction	1
1.1 The mechanisms of ageing	1
1.1.1 Hormesis	1
1.1.2 Caloric restriction	3
1.1.3 Oxidative stress and mitohormesis	4
1.1.4 Protein folding homeostasis and ageing	7
1.2 The model organism <i>Caenorhabditis elegans</i>	9
1.3 Compounds and their influence on ageing	12
1.3.1 Punicalagin	12
1.3.2 RO 90-7501	13
1.4 Aim of the thesis	14
2. Material and experimental procedures	15
2.1 Material	15
2.1.1 Chemicals	15
2.1.2 Growth media	15
2.1.3 Bacterial media	15
2.1.4 Buffers and solutions	16
2.2 Experimental procedures	19
2.2.1 <i>Caenorhabditis elegans</i> strains and maintenance	19

2.2.2 <i>Escherichia coli</i>	19
2.2.3 Compound treatment	20
2.2.4 Treatment of <i>C. elegans</i>	21
2.2.5 Synchronisation	21
2.2.6 Lifespan analyses	22
2.2.7 Locomotion assay	22
2.2.8 Pumping rate.....	23
2.2.9 Paraquat stress resistance assay	23
2.2.10 Thermotolerance stress resistance assay	23
2.2.11 Determination of ATP	24
2.2.12 Respiration assay.....	25
2.2.13 Mitochondrial ROS levels.....	25
2.2.14 Amplex Red-based determination of hydrogen peroxide levels	26
2.2.15 Antioxidant enzyme activities	27
2.2.16 Glucose oxidation assay	28
2.2.17 Fat content	28
2.2.18 Paralysis assay	29
2.2.19 Isolation of mitochondria from rat liver and respiration measurement	30
2.2.20 Complex I activity assay with mitochondria from mouse liver	32
2.2.21 Protein determination	33
2.2.22 Statistical analyses.....	33
3. Results	34
3.1 Modulation of life expectancy of <i>C. elegans</i> by punicalagin and RO 90-7501	34
3.2 The hydrolysis product of punicalagin does not influence life expectancy of <i>C. elegans</i>	35
3.3 Punicalagin and RO 90-7501 increase locomotion of <i>C.elegans</i>	36

3.4	Ro 90-7501 induces stress resistance against oxidative and thermal stress.....	38
3.5	Influence of punicalagin and RO 90-7501 on oxygen consumption	40
3.6	Complex I activity of isolated mouse mitochondria under the influence of punicalagin and RO 90-7501	41
3.7	Respiration of isolated rat mitochondria under the influence of punicalagin and RO 90-7501	43
3.8	Influence of punicalagin and RO 90-7501 on ATP levels of <i>C. elegans</i>	44
3.9	Punicalagin decreases the glucose oxidation in <i>C. elegans</i>	46
3.10	Fat content and pumping rate of <i>C. elegans</i> are not influenced by punicalagin	47
3.11	AMPK/AAK-2 is involved in lifespan extension of <i>C. elegans</i> under the influence of punicalagin and RO 90-7501.....	48
3.12	Modulation of ROS formation in <i>C. elegans</i> by punicalagin and RO 90-7501	50
3.13	The lifespan-extending effect of punicalagin and RO 90-7501 in <i>C. elegans</i> is abolished by supplementation of an antioxidant	52
3.14	P38 MAPK/PMK-1 is involved in lifespan extension of <i>C. elegans</i> ..	53
3.15	The lifespan extension of <i>C. elegans</i> is mediated by Nrf2/SKN-1....	55
3.16	The role of FOXO/DAF-16 for lifespan extension of <i>C. elegans</i>	60
3.17	The modulation of antioxidant enzyme activities in <i>C. elegans</i> by punicalagin and RO 90-7501	61
3.18	Punicalagin and RO 90-7501 modulate the protein homeostasis in <i>C. elegans</i>	64
3.19	The UPR _{ER} is involved in punicalagin- and RO 90-7501-mediated longevity of <i>C. elegans</i>	68
4.	Discussion	72
5.	Conclusion and perspectives	92

Appendices	XIII
I Strains used.....	XIII
II Statistical analyses of lifespan assays.....	XV
References	XVII
Acknowledgements	XXXIII
Selbstständigkeitserklärung	XXXIV

Summary

Compounds that delay ageing and prevent against age-related diseases in model organisms have potential application in promoting a healthy lifespan in humans.

In this work, the roundworm, *Caenorhabditis elegans*, was exposed to punicalagin, a natural ellagitannin from the pomegranate, and to RO 90-7501, a pharmaceutical compound previously identified to diminish Alzheimer-related A β ₄₂-induced toxicity. Punicalagin significantly increased mean lifespan of *C. elegans* by 7.41 %, while RO 90-7501 had an effect of 6.16 %. Furthermore, RO 90-7501 significantly increased survival rate under both thermal and oxidative stress conditions. Both compounds inhibit complex I of the electron transport chain, resulting in significantly impaired respiration after 6 h of application, a short-term energy deficit, and a transiently increased release of mitochondrial-derived ROS. The energy-sensing kinase AMPK/AAK-2 in response promotes mitochondrial metabolism following short-term metabolic stress, which leads to subsequently compensated or even elevated respiration rates and ATP levels after long-term exposure to both compounds.

The initially elevated formation of mitochondrial-derived ROS is sensed by p38 MAPK/PMK-1 and activates the transcription factor Nrf2/SKN-1 and FoxO/DAF-16 as well as the heat shock factor HSF1/HSF-1, which then initiate downstream effects resulting in an adaptive response. This induction of stress defense can be attributed to the upregulation of antioxidant enzymes including superoxide dismutase and/or catalase as well as the improvement of proteostasis and UPR. The adaptive response then terminates the initial ROS signal and in parallel extends lifespan of *C. elegans*, all of which is abolished by antioxidant supplementation.

Taken together, punicalagin and RO 90-7501 are capable of extending lifespan and decelerate ageing in the roundworm *C. elegans* and may be considered candidate compounds to prevent against ageing and age-related diseases in higher organisms possibly including humans.

Zusammenfassung

Substanzen, welche den Alterungsprozess verlangsamen können und präventiv bei altersassoziierten Erkrankungen wirken, sind von großem wissenschaftlichen Interesse für die Förderung gesunden Lebens des Menschen.

In der vorliegenden Arbeit wurde der Fadenwurm *Caenorhabditis elegans* mit Punicalagin, einem Ellagitannin aus dem Granatapfel sowie RO 90-7501, einem Pharmazeutikum, das Alzheimer-bedingte A β ₄₂-induzierte Toxizität *in vitro* verringern kann, exponiert. Punicalagin konnte die mittlere Lebenserwartung von *C. elegans* signifikant um 7,41 %, RO 90-7501 um 6,16 % erhöhen. Zudem zeigte sich unter Behandlung mit RO 90-7501 eine signifikant höhere Überlebensrate sowohl unter oxidativem als auch unter thermischem Stress. Beide Substanzen hemmen die mitochondriale Atmungskette an Komplex I. Dies hat eine signifikante Verringerung der Respiration nach 6-stündiger Behandlung, ein kurzzeitiges Energiedefizit und einen temporären Anstieg der Bildung mitochondrialer ROS zur Folge. Der kurzzeitige metabolische Stress führt zu einer AMPK/AAK-2-abhängigen Förderung des mitochondrialen Metabolismus. Dies resultiert schließlich in kompensierter oder aber signifikant erhöhter Respirationsrate und ATP-Bildung nach längerer Behandlung mit beiden Substanzen. Das anfänglich verstärkte ROS-Signal aktiviert, p38 MAPK/PMK-1-vermittelt, die Transkriptionsfaktoren Nrf2/SKN-1 und FoxO/DAF-16 sowie den Hitzeschockfaktor HSF1/HSF-1, welche nachgeschaltete Reaktionen auslösen, die schlussendlich eine adaptive Antwort hervorrufen. Diese Induktion der Stressabwehr kann auf die erhöhte Aktivität der antioxidativen Enzyme Katalase und/ oder Superoxiddismutase, aber auch auf die Verbesserung der Proteostase und UPR zurückgeführt werden. Die Stressantwort beendet schließlich das anfänglich erhöhte mtROS-Signal und führt parallel zu einer Lebensverlängerung von *C. elegans*, welche durch zusätzlich zugeführte Antioxidantien aufgehoben wurde.

Die Ergebnisse zeigen, dass Punicalagin und RO 90-7501 die Lebenserwartung von *C. elegans* erhöhen und möglicherweise auch präventiv gegen Alterung und altersassoziierte Erkrankungen in anderen Organismen und dem Menschen wirken könnten.

List of abbreviations

°C	degree celsius
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
µmol	micromole
ADP	adenosine diphosphate
AIP1	actin-interacting protein 1
AMP	adenosin-5'-monophosphate
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATP	adenosintriphosphate
Aβ	beta-amyloid
BCA	bicinchoninic acid
BHA	butylated hydroxyanisole
BiP	immunoglobulin heavy chain-binding protein
BSA	bovine serum albumin
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CGC	Caenorhabditis genetics center
CO ₂	carbon dioxide
CoA	coenzyme A
cpm	count(s) per minute
CR	caloric restriction
CTL	catalase
ctrl	control
d	day(s)
<i>D.melanogaster</i>	<i>Drosophila melanogaster</i>
DCIP	2,6 dichlorophenol-indophenol
ddH ₂ O	double-distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DYT	double yeast trypton
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	for example
EA	ellagic acid
ER	endoplasmatic reticulum
ETC	electron transport chain
FCCP	carbonylcyanid-p-trifluormehoxyphenyl-hydrazone
FMN	flavin mononucleotide
FoxO	forkhead box protein O
g	gram(s)
GST	glutathione-s-transferase
h	hour(s)
H ₂ O ₂	hydrogen peroxide
HepG2	human liver carcinoma cell line
HRP	horse-radish peroxidase
HSF1	heat shock factor 1
HSP	heat shock protein
IFN-β	interferon beta

IGF	insulin-like growth factor
IRE1	inositol-requiring protein 1
KO	knock-out
l	litre(s)
L4	L4 larval stage
LB	Luria-Bertani (broth)
M	molar
MAPK	mitogen-activated proteinkinase
min	minute(s)
ml	milliliter(s)
mM	millimolar
mmol	millimole
mRNA	messenger RNA
mtROS	mitochondrial-derived ROS
MW	molecular weight
n	number of replicates
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NGM	nematode growth media
nmol	nanomole
Nrf2	NF-E2 [nuclear factor (erythroid-derived 2)] related factor-2
O ₂	dioxygen
O ₂ ⁻	superoxide radical
p	p-value
p38	protein 38
PERK	protein kinase RNA-like endoplasmic reticulum kinase
pmol	picomole
Punic	Punicalagin
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RO 90	RO 90-7501
ROS	reactive oxygen species
Rot	rotenone
rpm	round(s) per minute
s	second(s)
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SD	standard deviation
SEM	standard error of the mean
SOD	superoxide dismutase
TLR3	Toll-like receptor 3
TOR	target of rapamycin
U	unit(s)
UPR	unfolded protein response
UV	ultraviolet
wt	wild type
XPB1	X-box binding protein 1

List of figures

Figure 1: Non-linear dose-response curve of a treatment with a hormetic effect	2
Figure 2: Generation of ROS in the mitochondria and their elimination by cellular antioxidants.....	5
Figure 3: Images of an adult hermaphrodite	9
Figure 4: Life cycle of <i>C. elegans</i> at 22 °C	10
Figure 5: Structure of punicalagin and ellagic acid	13
Figure 6: Structure of RO 90-7501	13
Figure 7: Procedure for the treatment of <i>C. elegans</i>	21
Figure 8: The luciferase reaction.....	25
Figure 9: Principle of the SOD determination	27
Figure 10: Timetable for the Paralysis assay	30
Figure 11: Procedure for the isolation of mitochondria from liver	31
Figure 12: Punicalagin extends lifespan of <i>C. elegans</i>	34
Figure 13: RO 90-7501 extends lifespan of <i>C. elegans</i>	35
Figure 14: Ellagic acid has no influence on lifespan of <i>C. elegans</i>	36
Figure 15: Punicalagin increases locomotion of <i>C. elegans</i> after a 10 d treatment.....	37
Figure 16: RO 90-7501 increases locomotion of <i>C. elegans</i> after an 8 d treatment.....	37
Figure 17: RO 90-7501 increases resistance against oxidative stress in <i>C. elegans</i>	38
Figure 18: RO 90-7501 increases resistance against thermal stress in <i>C. elegans</i>	39
Figure 19: Modulation of oxygen consumption by punicalagin.....	40
Figure 20: Modulation of oxygen consumption by RO 90-7501	41
Figure 21: Complex I activity of isolated mouse mitochondria is inhibited by punicalagin.....	42
Figure 22: Complex I activity of isolated mouse mitochondria is inhibited by RO 90-7501.....	42
Figure 23: Respiration of isolated rat mitochondria is inhibited by punicalagin	43

Figure 24: Respiration of isolated rat mitochondria is inhibited by RO 90-7501	44
Figure 25: Modulation of ATP levels in <i>C. elegans</i> by punicalagin.....	45
Figure 26: Modulation of ATP levels in <i>C. elegans</i> by RO 90-7501	45
Figure 27: Punicalagin decreases glucose oxidation in <i>C. elegans</i>	46
Figure 28: Punicalagin has no influence on fat content of <i>C. elegans</i>	47
Figure 29: Pumping rate of <i>C. elegans</i> is not influenced by punicalagin	48
Figure 30: Lifespan extension mediated by Punicalagin is AAK-2-dependent .	49
Figure 31: Lifespan extension mediated by RO 90-7501 is AAK-2-dependent	49
Figure 32: Modulation of mtROS formation in <i>C. elegans</i> after treatment with punicalagin.....	50
Figure 33: Modulation of H ₂ O ₂ formation in <i>C. elegans</i> after treatment with punicalagin.....	51
Figure 34: Modulation of mtROS formation in <i>C. elegans</i> after treatment with RO 90-7501.....	51
Figure 35: BHA abolishes the effect of punicalagin on lifespan of <i>C. elegans</i> .	52
Figure 36: BHA abolishes the effect of RO 90-7501 on lifespan of <i>C. elegans</i>	53
Figure 37: Punicalagin-induced longevity of <i>C. elegans</i> requires PMK-1	54
Figure 38: RO 90-7501-induced longevity of <i>C. elegans</i> requires PMK-1	54
Figure 39: Lifespan extension of <i>C. elegans</i> by punicalagin is mediated by <i>skn-1</i> (zu135).....	55
Figure 40: Lifespan extension of <i>C. elegans</i> by RO 90-7501 is mediated by <i>skn-1</i> (zu135)	56
Figure 41: Lifespan extension of <i>C. elegans</i> by punicalagin is mediated by <i>skn-1</i> (zu67).....	57
Figure 42: Lifespan extension of <i>C. elegans</i> by RO 90-7501 is mediated by <i>skn-1</i> (zu67)	57
Figure 43: Lifespan extension of <i>C. elegans</i> by punicalagin is not mediated by <i>skn-1</i> (zu135 /nT1[qls51];gels10[ges-1p::skn-1c::gfp; rol-6(su1006)).....	58
Figure 44: Lifespan extension of <i>C. elegans</i> by RO 90-7501 is not mediated by <i>skn-1</i> (zu135 /nT1[qls51];gels10[ges-1p::skn-1c::gfp; rol-6(su1006)).....	59
Figure 45: Lifespan extension mediated by punicalagin is not DAF-16-dependent.....	60

Figure 46: Lifespan extension mediated by RO 90-7501 is DAF-16-dependent	61
Figure 47: Punicalagin does not influence SOD activity of <i>C. elegans</i>	62
Figure 48: Treatment with punicalagin induces the catalase activity of <i>C. elegans</i>	62
Figure 49: RO 90-7501 induces the SOD activity of <i>C. elegans</i>	63
Figure 50: Treatment with RO 90-7501 induces the catalase activity of <i>C. elegans</i>	63
Figure 51: Punicalagin slows protein aggregation in a transgenic <i>C. elegans</i> model of human proteotoxic disease.....	64
Figure 52: RO 90-7501 slows protein aggregation in a transgenic <i>C. elegans</i> model of human proteotoxic disease.....	65
Figure 53: Lifespan extension of <i>C. elegans</i> induced by punicalagin is HSF-1-dependent.....	66
Figure 54: Lifespan extension mediated by RO 90-7501 is HSF-1-dependent	66
Figure 55: Lifespan extension of <i>C. elegans</i> mediated by punicalagin is AIP-1-dependent.....	67
Figure 56: Lifespan extension mediated by RO 90-7501 is AIP-1-dependent..	68
Figure 57: Lifespan modulation of UPR _{ER} KO mutants of <i>C. elegans</i> after punicalagin treatment.....	69
Figure 58: Lifespan modulation of UPR _{ER} KO mutants of <i>C. elegans</i> after RO 90-7501 treatment	70
Figure 59: Mechanism of punicalagin actions in <i>C. elegans</i>	93
Figure 60: Mechanism of RO 90-7501 actions in <i>C. elegans</i>	94

List of tables

Table 1: Strains used XIII

Table 2: Results and statistical analyses of lifespan assays modulated by
punicalagin.....XV

Table 3: Results and statistical analyses of lifespan assays modulated by
RO 90-7501.....XVI

1. Introduction

1.1 The mechanisms of ageing

Biological ageing (senescence) is described as the disruption of molecular and cellular changes with the passage of time leading to death. In general, ageing, senescence and death are the final manifestations of unsuccessful homeostasis (Rattan 2006). The limitation of the homeodynamic property is the response and adaption to stress. A stressor is an environmental, chemical or biological factor leading to a series of events in the organism. A response to low doses of stress by adapting can improve the homeodynamics of an organism while a failed response and adaption leads to damage and harmful effects like an increasing risk of age-related diseases (Rattan 2008). Scientists all over the world are interested in the ageing process, the molecular regulation and the question, how ageing can be influenced and life expectancy can be increased. There are two models which had been discussed and are well established now, hormesis and caloric restriction. Both are interventions to extend lifespan of organisms without genetic manipulation.

1.1.1 Hormesis

Hormesis describes the adaptive response to low exposures of stress. In toxicology it is described as low-dose stimulation and a high-dose inhibition (Calabrese 2002). The story began in the early 1880s, when the pharmacologist Hugo Schulz found out that low doses of toxins were able to stimulate the growth of yeast. Together with the findings of Rudolph Arndt who studied animals given low doses of drugs, the Arndt-Schulz rule was established (Schulz and Crump 2003). The term “hormesis” was coined by Southam & Ehrlich (Southam and Ehrlich 1943). The dose-response curve is neither threshold nor linear, it is more a non-linear U- or inverted U-shaped form (Calabrese 2006) (Figure 1).

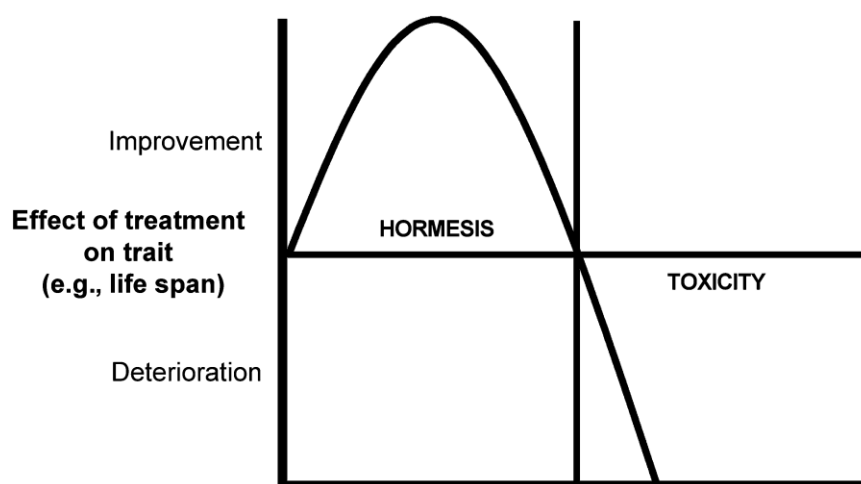


Figure 1: Non-linear dose-response curve of a treatment with a hormetic effect
(Gems and Partridge 2008)

Recent studies discuss whether the U-shaped form is misleading to describe the hormesis response; hormesis seems to be more asymmetric and a J shaped form fits better because of showing that the low-dose effect is much milder than the high-dose side of the curve (Douglas 2008). There is a huge variety of chemical and biological agents that show hormetic dose-response in different organisms.

For example, vitamins (Tuohimaa et al. 2004), components of plants or food (Moriguchi et al. 1996), trace elements (Mocchegiani et al. 2008), minerals, ethanol (Kurta and Palestis 2010), or even insecticides and pesticides (Kociba et al. 1978; Sukata et al. 2002), heavy metals (Helmcke and Aschner 2010), antibiotics, chemotherapeutics (Calabrese 2005) and pro-oxidants (Martinez-Sanchez et al. 2011) are natural or synthetic compounds showing hormetic effects (Ali and Rattan 2006; Hayes 2009).

Hormesis in ageing is defined as the life supporting beneficial effects exhibited by repeated mild exposure to stress through induction of cellular responses and repair systems (Rattan 2004). Therefore, mild and periodic stresses can prevent ageing and age-related diseases and increase lifespan. Besides natural and synthetic compounds at low doses, exercise (Radak et al. 2005), radiation (Lamb and Dyer 1971), hypergravity (Minois 2006), heat stress (Macario and Conway de Macario 2002), or caloric restriction (de Oliveira et al. 2003) are known interventions to exhibit mild stresses to support healthy ageing and increase lifespan.

1.1.2 Caloric restriction

Caloric restriction (CR) is a dietary regimen that restricts calorie intake by about 30 - 50 %. It is the most commonly used intervention to decelerate biological ageing and attenuate age-related diseases, resulting in an increase of median and maximum lifespan in various species (Anderson et al. 2009). As early as 1935, McCay described the lifespan-extending effect of caloric restriction in rats (McCay et al. 1935). Although CR showed an extended lifespan in yeast (Kryakov et al. 2012), insects (Bross et al. 2005), nematodes (Schulz et al. 2007), rats (Abalan et al. 2010), mice (Sun et al. 2009), and monkeys (Colman et al. 2009), the anti-ageing effect in humans is still discussed and a matter of interest. It is clear that CR has beneficial and health-promoting effects in humans such as reducing risk factors for age-related diseases such as type 2 diabetes, cancer (Harvie and Howell 2012), or cardiovascular (Fontana et al. 2004) and neurodegenerative diseases (Arumugam et al. 2006) and improving DNA-repair parameters (Raji et al. 1998), but it is still debated whether CR is able to increase life expectancy in primates because a recent study disproved lifespan extension in monkeys (Mattison et al. 2012).

The mechanisms behind CR are controversial. One possible mechanism is the CR-mediated longevity through sirtuins. Sirtuins are NAD(+)-dependent histone deacetylases influencing ageing and regulating transcription, apoptosis, and stress resistance (Morris 2012). The involvement of sirtuins in the longevity mediated through CR is well supported (Tissenbaum and Guarente 2001), but also disputed (Houthoofd and Vanfleteren 2006). Another hypothesis is the TOR (target of the rapamycin) signaling pathway. TOR kinase is induced by nutrients and influences cell mass growth and cell division. Therefore, TOR drives cellular ageing (Blagosklonny 2010). The inhibition of TOR extends lifespan in fruitflies (Kapahi et al. 2004), worms (Vellai et al. 2003), and mice (Sharp and Bartke 2005). A connection between CR and TOR was also found. Metformin, which mimics CR and inhibits TOR via AMPK, can prolong lifespan in cancer-prone mice (Anisimov et al. 2008). Another studied mediator for the anti-ageing activity of caloric restriction is IGF1 (insulin-like growth factor 1) (Gems and Partridge 2001). Genetic alterations of the insulin/IGF1 signaling pathway extend lifespan in *C. elegans* (Lin et al. 2001), *Drosophila melanogaster* (Tatar et al. 2001), and mice (Flurkey et al. 2001). There are a

few studies connecting the insulin/IGF1 signaling pathway to CR (Vanfleteren and Braeckman 1999; Shimokawa et al. 2008). Another possible explanation is the ability of CR to reduce oxidative stress mediated through reactive oxygen species (ROS). The restricted caloric uptake leads to decreased metabolic activities with decreasing formation of ROS (Sohal and Weindruch 1996). This theory was disproved by Masoro et al. (Masoro et al. 1982) and Houthoofd et al. (Houthoofd et al. 2002). Hormesis may explain the beneficial effects of CR. Caloric restriction is a low-intensity stressor (Parsons 2000; Yu and Chung 2001) working through pathways involved in stress response, molecular damage prevention and metabolic regulation. Recent studies deal with the question whether CR decreases metabolic rate. Findings in *C.elegans* suggest that CR is increasing the metabolic rate (Schulz et al. 2007), contrarily, in *Drosophila melanogaster* no positive correlation between enhanced lifespan and decreased metabolism was observed (Hulbert et al. 2004). It is clear that CR is an important intervention for the prevention of age-related diseases and increasing life expectancy. It can induce stress-response mechanisms like radical-scavenging and phase II response enzymes (Semsei et al. 1989; Rao et al. 1990). This link between increased antioxidant capacity and CR led to the argument, that CR primarily decreases ROS production. Recent findings argue against this hypothesis showing that CR initiates beneficial alterations like increased metabolic rate and ROS production leading to adaptive response mechanisms (Schulz et al. 2007; Sharma et al. 2011) and an increased lifespan.

1.1.3 Oxidative stress and mitohormesis

Oxidative stress results from increasing levels of ROS during environmental stress; e.g., thermal or UV exposure or even exposure to natural and synthetic compounds. The balance between oxidants and antioxidants is shifted to oxidants in cases of oxidative stress. Reactive oxygen species are produced by living organisms as a result of normal cellular metabolism and environmental factors. They are generated within the mitochondria during the transport of electrons through a mitochondrial electron transport chain of enzymatic complexes during respiration (Nakano et al. 2006) (Figure 2).

They are highly reactive molecules and can damage cell structures. The most important ROS are the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) (Birben et al. 2012). Under normal physiological conditions, ROS are rapidly eliminated by antioxidant enzymes, such as SOD or CTL (Figure 2).

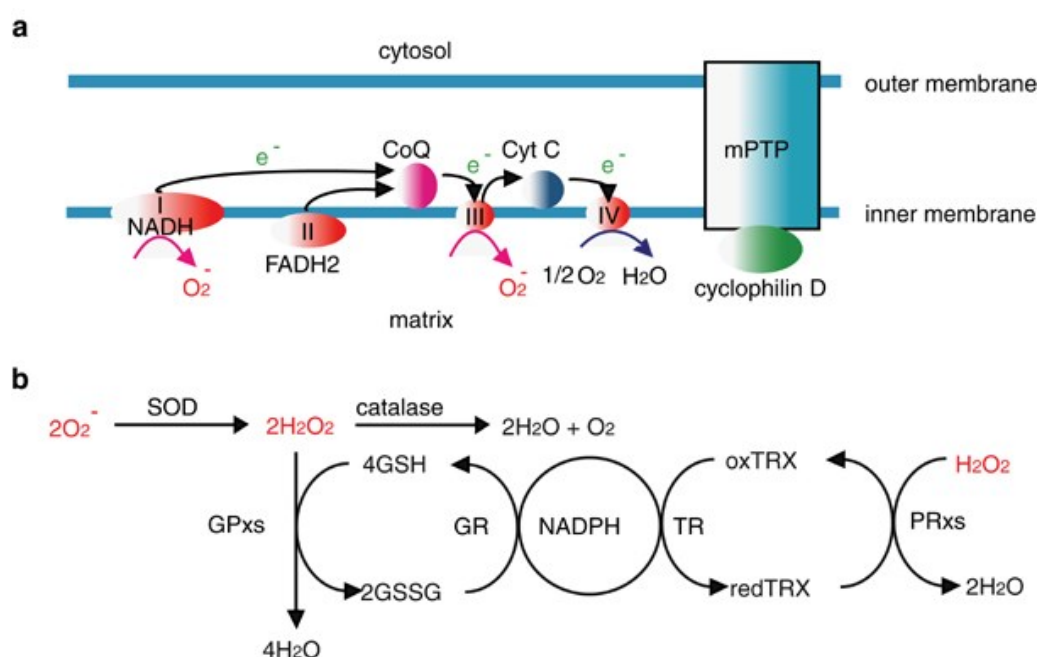


Figure 2: Generation of ROS in the mitochondria and their elimination by cellular antioxidants

a) Formation of mtROS at the electron transport chain within the mitochondria

b) Elimination of mtROS by cellular antioxidants

(Nakano et al. 2006)

Therefore, the metabolic rate is linked to the formation of ROS. In 1908, Max Rubner first noted the inverse correlation between the metabolic rate (oxygen consumption) and longevity (Rubner 1908). This was later expanded by Pearl in his rate-of-living hypothesis, stating that lifespan is strictly an inverse function of metabolic rate (Pearl 1928). This hypothesis is no longer accepted, but it was the first hint linking the ageing process to oxidative metabolism. This theory was improved by Denham Harman proposing that ROS are formed from normal metabolic processes and play an important role in ageing (Harman 1956). This free radical theory was modified in 1972 giving the mitochondria the central role for ageing, the mitochondrial free radical theory (Harman 1972). It was later found, that not only free radicals, but also peroxides and aldehydes play an

important role in provoking oxidative damage to cells, which led to the oxidative stress theory of ageing. Because of an imbalance between oxidants and antioxidants, there is a chronic state of oxidative stress in organisms under normal conditions (Sohal and Weindruch 1996). The accumulation of oxidative damage increases during ageing and leads to a progressive loss of cellular function. Reactive oxygen species are a byproduct of oxidative phosphorylation taking place in the mitochondria. Therefore, beside the generation of ATP, mitochondria seem to play a central role in physiological and pathophysiological processes (Ristow and Schmeisser 2011). Mitochondrial dysfunction could lead to age-related diseases like cancer (Ristow 2006), diabetes (Wiederkehr and Wollheim 2006), neurodegenerative diseases (Tatsuta and Langer 2008) and can be seen as the main cause for ageing (Trifunovic and Larsson 2008). Conversely, improving the mitochondrial function could decelerate the ageing process, thereby preventing age-related diseases and increasing life expectancy. There are few studies showing the effect of CR is increasing mitochondrial metabolism (oxygen consumption) and extending the lifespan of organisms (Schulz et al. 2007; Birben et al. 2012; Ocampo et al. 2012). The same can be shown for physical exercise (Schulz et al. 2010), impairment of mTOR pathway (Powers et al. 2006), reduced insulin/IGF1 signaling (Zarse et al. 2012) and pharmacological treatments (Schmeisser et al. 2011). Following the free radical theory of ageing by Harman, increased metabolic rate leading to the increased formation of ROS is the limiting factor for lifespan (Shimokawa et al. 2008). Therefore, interventions that can reduce oxidative stress have long been of scientific interest (Moskovitz et al. 2001; Harrington and Harley 1988). However a few studies could not find the health-promoting effect with supplementation of e.g. antioxidants (Sesso et al. 2008; Liu et al. 1999). These findings questioned the free radical theory of ageing. Reactive oxygen species could possibly play an important role in cellular signaling promoting health (Veal et al. 2007; Ristow et al. 2009). Therefore, low levels of ROS may lead to adaptive processes like stress resistance and longevity via the activation of stress defensive mechanisms culminating in the promotion of health and longevity (Masoro 1998; Yu and Chung 2001). Because high doses of ROS are still damaging and destructing, ROS seem to show hormetic effects with low-dose stimulation and high-dose inhibition.

In regard to the formation of ROS in the mitochondria, the term “mitohormesis” or mitochondrial hormesis was coined (Tapia 2006). Many studies support this hypothesis by showing that increasing metabolic rate leads to enhanced stress resistance and longevity (Vanfleteren and De Vreese 1995; Nazarewicz et al. 2007; Sharma et al. 2011).

In summary, interventions that can induce mitochondrial activity accompanied by low-dose stimulation of ROS (mitohormesis) like exercise, caloric restriction, reduced insulin/IGF1 signaling pathway and pharmacological treatments can lead to adaptive response mechanisms and finally to longevity. Mitohormesis is a possible mechanism to influence ageing and age-related diseases in a positive way.

1.1.4 Protein folding homeostasis and ageing

There is evidence for the role of unfolded/misfolded proteins in ageing and the occurrence of age-related diseases. During normal ageing, a decay of chaperoning systems, functionality of repair enzymes, and the silencing of the heat shock response leads to protein misfolding, accumulation, and aggregation and to loss-of-function or gain-of-function toxicity (Macario and Conway de Macario 2002). In the case of age-related cognitive dysfunctions proteins are transformed from their soluble form to insoluble fibrils or aggregated plaques leading to conformational disorders like Alzheimer’s disease, Parkinson’s disease, or type 2 diabetes mellitus (Brown and Naidoo 2012). An efficient protein folding homeostasis, or proteostasis, has an important role in the health of the cells and the lifespan of an organism. Proteostasis guarantees folding, adjustment of newly-synthesized proteins, repair of damaged proteins, and clearance (Kikis et al. 2010). In cases of stress or even normal metabolic events during respiration, glycolysis or ageing, protein damage can arise. In healthy cells, all processes are balanced by molecular chaperones, detoxifying enzymes, degradation machinery and adaptive stress response (Parsell and Lindquist 1993).

Oxidative modifications of proteins are one source of damage. Reactive oxygen species can damage DNA, lipids, and proteins (Stadtman and Oliver 1991). Glycolytic intermediates can also modulate proteins and form advanced glycation end products (AGEs) (Lo et al. 1994). Another possibility for damaging

proteins are genetic mutations and polymorphisms, genomic instability, and mistranslation (Pakula and Sauer 1989). To protect the cell from misfolded and damaged proteins in proteotoxic situations, there are adaptive transcriptional stress responses as the cytoplasmatic heat shock response and the unfolded protein response (UPR) (Westerheide and Morimoto 2005; Brown and Naidoo 2012). The endoplasmatic reticulum (ER) contains such an UPR system, which is triggered by the accumulation of misfolded proteins in the ER (Berridge 2002). Under conditions of stress, like glucose/energy deprivation, redox changes or viral infections, protein folding can be disrupted leading to the accumulation of unfolded proteins (Kaufman et al. 2002). The UPR triggers different protective cellular responses: the upregulation of ER chaperones such as BIP/GRP78 for the refolding of proteins, the reduction in protein translation mediated through PERK and the degradation of misfolded proteins by the proteasom. Additional PERK activation also up-regulates antioxidant stress response through Nrf2 (Johnson et al. 2008; Brown and Naidoo 2012).

The transcription factor HSF1 mediates the activation of transcription and translation of genes coding for molecular chaperones (heat shock proteins) of the quality control machinery maintaining the structural integrity of proteins (Barna et al. 2012). HSF1 and the components of the UPR signaling and other stress-inducible chaperones are essential for life and development and are therefore necessary for the maintenance of proteostasis under normal conditions (Xiao et al. 1999; Reimold et al. 2001). There is evidence that genetic pathways regulating lifespan suppress proteotoxicity. In *C. elegans* HSF-1 is required for the *daf-2*-insulin/IGF1 receptor mutation to extend lifespan (Hsu et al. 2003). The inactivation of DAF-16 and HSF-1 accelerate polyQ protein aggregation. Insulin-like signaling could influence proteostasis through the activation of DAF-16 and HSF-1 (Hsu et al. 2003; Morley and Morimoto 2004).

1.2 The model organism *Caenorhabditis elegans*

Typical invertebrate model organisms to study lifespan and ageing include the fruit fly *Drososophila melanogaster* (Sondhi 1968; Helfand and Rogina 2003), the yeast *Saccharomyces cerecisiae* (Jazwinski 1993), and the nematode *Caenorhabditis elegans* (Gershon and Gershon 2002).

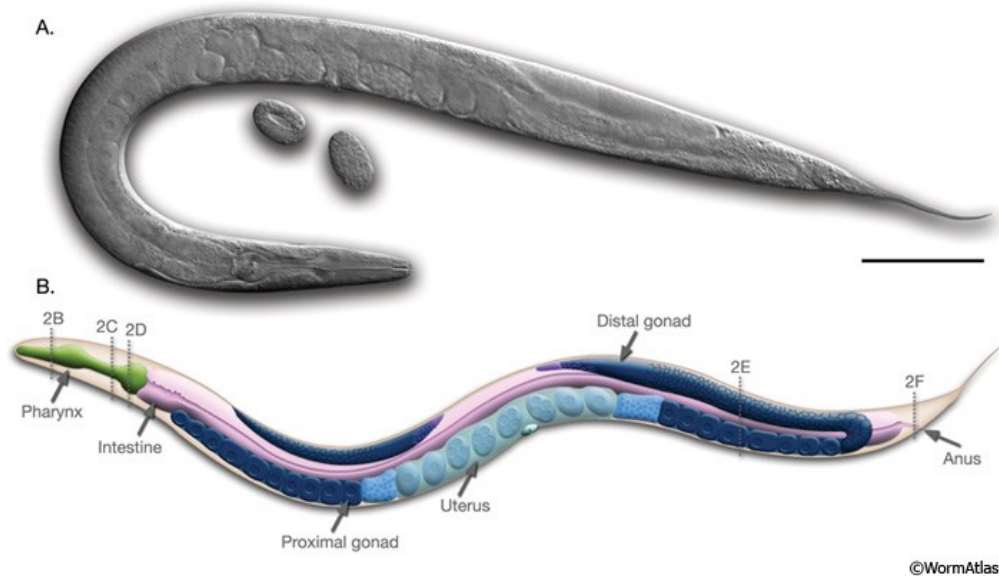


Figure 3: Images of an adult hermaphrodite

A. DIC image of an adult hermaphrodite, left lateral side

B. Schematic drawing of anatomical structures, left lateral side

(www.wormatlas.org)

Caenorhabditis elegans is an ubiquitous, small (1 - 1.5 mm of length), free-living nematode (Figure 3) originally derived from a fungal compost in the late 1950s (Dougherty and Hansen 1956) and later introduced as the wild type N2 strain raised under laboratory conditions (Brenner 1974). It is generally cultured monoxenically on bacterial lawns of *E. coli*. These bacterial cells cover all the nutritional requirements of *C. elegans* except for sterols, which are additionally provided by cholesterol in the NGM agar. The nematode is a hermaphrodite producing around 0.2 % of males via X-chromosome non-disjunction (Hodgkin et al. 1979). These hermaphrodites reproduce by self-fertilization. *Caenorhabditis elegans* shows attributes similar to more developed organisms; a nervous system, an epidermis, an intestine and gonads (Figure 3). *Caenorhabditis elegans* is composed of 959 cells and a fixed number in each tissue. It undergoes four larval stages (Figure 4).

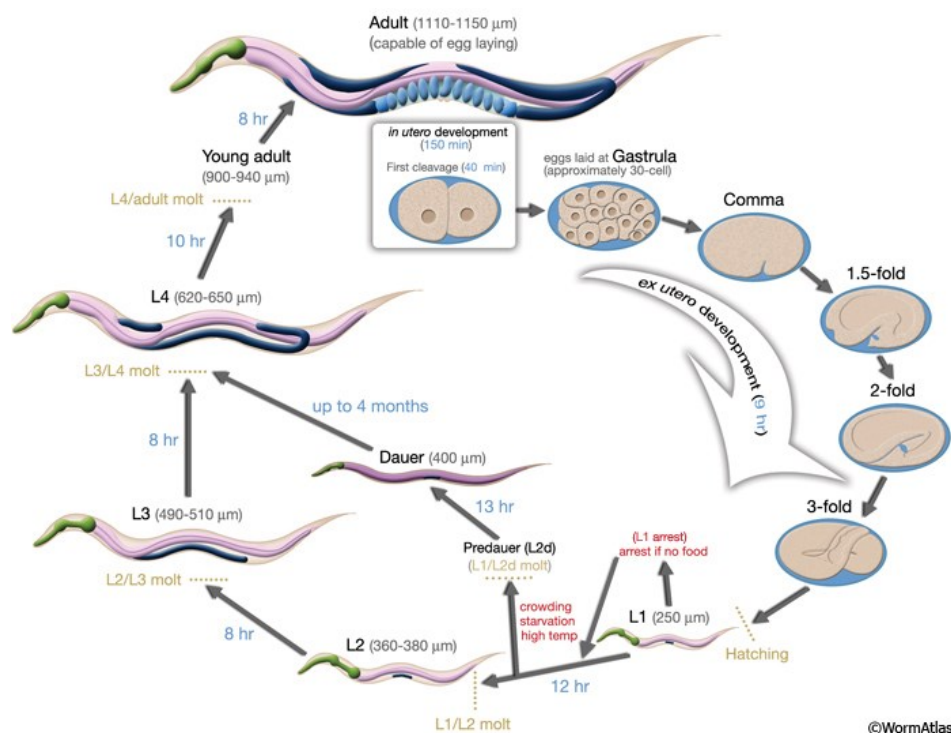


Figure 4: Life cycle of *C. elegans* at 22 °C

(www.wormatlas.org)

Under extreme environmental stress like thermal stress, oxygen deprivation, or food deprivation, the nematode in the L2 larval stage can form a Dauer larvae with highly effective stress resistance mechanisms (Riddle and Albert 1997). In this form, they can survive 8 - 10 times longer than normal worms. When the environmental conditions improve, they can exit the Dauer larval stage and develop normally.

The investigation of this huge lifespan extension in Dauer larvae led to the exploration of genes and pathways in ageing research (Kenyon et al. 1993; Riddle and Albert 1997). *Caenorhabditis elegans* fits as a model organism for scientific questions and the research of ageing and longevity. Nematodes have a high degree of cell differentiation and specialization. *Caenorhabditis elegans* is a relatively simple multi-cellular organism with 959 somatic cells in the full-grown hermaphrodite being essentially post-mitotic like most somatic cell systems in mammals. Nematodes can be maintained under inexpensive laboratory conditions; they have a short mean and maximum lifespan as well as a generation time of a few days. It is one of the simplest organisms with a

nervous system containing of 302 neurons (Watts and Strogatz 1998). Their genome is completely sequenced and genetic analyses is possible (C. Elegans Sequencing Consortium 1998). Many *C. elegans* genes have orthologs in higher organisms. For around 60 % of the human genes associated with diseases, a corresponding homolog can be found in *C. elegans* (C. elegans Sequencing Consortium 1998). A well-organized collection of strains and a genetic database is available to researchers. A complete knock-out of genes is possible and *C. elegans* mutant strains are also available. RNA interference by feeding the worms with transgenic bacteria is another useful and possible method for disrupting gene function. Scientists have been able to knock out 86 % of the around 20000 genes in the worm (Kamath et al. 2003). Nematodes have long been used as a model in ageing research. Many types of ageing studies were initiated with the finding that Dauer larvae is a time-out from ageing (Klass and Hirsh 1976). Genetic analyses of *C. elegans* started in 1980 (Johnson and Wood 1982), later, *age-1*, the first long-lived mutant was detected (Friedman and Johnson 1988) followed by *daf-2* being unable to form complete Dauer larvae (Kenyon et al. 1993).

Until now, there are a few methods known to delay ageing in *C. elegans* and thereby extend the lifespan. On one hand, it is possible to regulate ageing by genetic manipulation (Braeckman and Vanfleteren 2007), on the other hand CR is a suggested intervention in the ageing process. It can be achieved by supplementation of compounds blocking part of glucose metabolism (Schulz et al. 2007), by the use of less or no bacteria as food source (Hosono et al. 1989) or by genetic alterations like the *eat-2* mutant having a decreased pumping rate and limited food uptake (Lakowski and Hekimi 1998). Another possibility for altering the process of ageing is hormesis. Thereby well-dosed stressors are applied as chemicals, or hormesis is induced by physical stress like thermal stress (Michalski et al. 2001; Cypser and Johnson 2002). The effect of natural and synthetic compounds with health-beneficial and targeting effects on lifespan and ageing in *C. elegans* is well established (Wu et al. 2002; Wilson et al. 2006).

1.3 Compounds and their influence on ageing

There is ample evidence that natural and synthetic compounds can influence ageing and age-related diseases. Besides the health-beneficial and targeting effects of these compounds, they are able to extend lifespan in different organisms and therefore slow down the ageing process (Collins et al. 2006; Driver and Georgeou 2003; Kitani et al. 2007). For this work, a cell-based screening was performed to identify substances having the ability to influence ageing via the induction of mitochondrial metabolism. Punicalagin as a natural compound from the pomegranate and RO 90-7501 as a pharmaceutical substance were found to induce mitochondrial activity and possibly influence the process of ageing.

1.3.1 Punicalagin

Punicalagin [2.3-(S)-hexahydroxydiphenoyl-4.6-(S,S)-gallagyl-d-glucose] is an ellagitannin belonging to the family of polyphenols. Polyphenols are aromatic compounds generated from plants to protect against predators. The health promoting effects of polyphenols have long been of scientific interest. Polyphenols have antioxidant properties (Lansky and Newman 2007; Frei and Higdon 2003) and protect against age-related diseases such as cancer (Dragsted et al. 1993), neurodegenerative diseases (Joseph et al. 1999; Singh et al. 2008), and cardiovascular diseases (Aviram and Rosenblat 2012). The ellagitannin punicalagin is the most abundant polyphenol in the pomegranate (*Punica granatum* L.). It can spontaneously hydrolyse into smaller phenolic compounds such as ellagic acid (Gil et al. 2000). With a molecular weight of 1084.71 g/mol, punicalagin is the largest polyphenol known from the pomegranate (Figure 5) and is responsible for most of the antioxidant and health-beneficial capacity of pomegranate juice (Heber 2011).

More than 2 g/l punicalagin can be found in the pomegranate juice and can be extracted from the husk by processing (Seeram et al. 2005). Pomegranate ellagitannins show antiatherosclerotic (Aviram and Dornfeld 2001), anticancer (Sharp and Bartke 2005) and antioxidant activities (Vanfleteren and De Vreese 1995). Furthermore, neuroprotective effects in a transgenic mouse model expressing a form of amyloid precursor protein that causes early-onset familial Alzheimer's disease had been identified (Hartman et al. 2006).

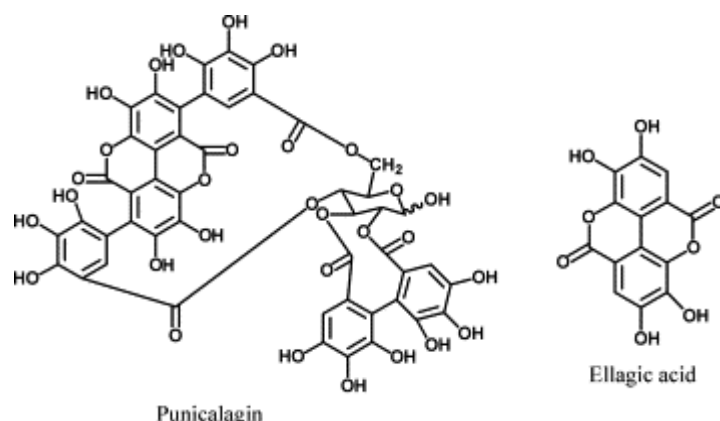


Figure 5: Structure of punicalagin and ellagic acid

(Seeram et al. 2005)

There are a few findings suggesting that punicalagin itself has health-promoting effects. It can suppress inflammatory cell signaling (Adams et al. 2006) and induce apoptosis in colon cancer cells by releasing ellagic acid (Larrosa et al. 2006). Furthermore, an immune-suppressive activity was detected (Lee et al. 2008). Punicalagin has high antioxidant activity (Vanfleteren and De Vreese 1995) but is non-toxic in rats given 6 % punicalagin-containing diet for 37 d (Cerdeira et al. 2003).

1.3.2 RO 90-7501

RO 90-7501 (2'-(4-aminophenyl)-[2,5'-bi-1H-benzimidazol]-5-amine) (Figure 6) is a synthetic molecule with a high content of π electrons. This compound was identified by a cell-based screening assay because of its ability to bind to mature $A\beta_{42}$ fibrils and to diminish the toxicity induced by $A\beta_{42}$ *in vitro* (Bohrmann et al. 2000). It can induce antiviral response by promoting TLR3 and RLR ligand-induced IFN- β gene expression via activation of p38 MAPK pathway (Guo et al. 2012).

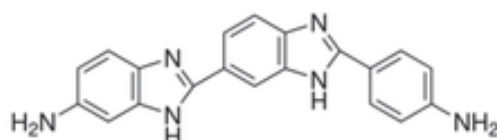


Figure 6: Structure of RO 90-7501

(Vanfleteren and Braeckman 1999)

1.4 Aim of the thesis

In this present work, the influence of two compounds, punicalagin and RO 90-7501, on the lifespan and stress resistance of *C. elegans* was investigated. Both compounds increased the production of ATP in a HepG2 cell-based screening. The increase in ATP is evidence for improved mitochondrial metabolism. Improving the mitochondrial function could decelerate the ageing process, thereby preventing age-related diseases and increasing life expectancy in a mitohormetic manner. The naturally occurring ellagitannin punicalagin, as well as the synthetic molecule RO 90-7501 were tested for their modulating effects on the metabolism of the nematode *C. elegans*. Besides studying both compounds in regards to lifespan and stress resistance, parameters reflecting the quality of life, such as locomotion or pumping rate, were examined. Furthermore, metabolic parameters of the nematodes, such as ATP production, respiration, and ROS formation were investigated. For the exploration of the underlying molecular mechanisms, specific genes, which are possibly involved in longevity and stress resistance induced by punicalagin and RO 90-7501, were detected by performing lifespan analyses with knock-out mutants for these genes. Finally, the modulation of metabolism of *C. elegans* by punicalagin and RO 90-7501 was analysed and discussed in the context of ageing and the mitohormesis concept.

2. Material and experimental procedures

2.1 Material

2.1.1 Chemicals

All chemicals were obtained from Sigma-Aldrich (Munich, Germany) unless stated otherwise.

2.1.2 Growth media

The nematodes were maintained on **NGM agar plates**. The following ingredients were necessary for 1000 ml:

NaCl (Applichem, Darmstadt, Deutschland)	3.0 g
Peptone (Fluka, Buchs, Schweiz)	2.5 g
Agar (SERVA, high gel-strength, Heidelberg, Deutschland)	17.0 g

1000 ml ddH₂O were added and the bottle was autoclaved.

After cooling the agar to 50 °C in a water bath the following ingredients were added:

1 M CaCl ₂	0.5 ml
5 mg/ml cholesterol in ethanol	1.0 ml
1 M MgSO ₄	1.0 ml
1 M potassium phosphate buffer (pH 6.0)	25.0 ml
Nystatin	5.0 ml

After mixing well, the plates were poured under a laminar flow hood.

2.1.3 Bacterial media

DYT media was prepared by adding the following ingredients to 1000 ml ddH₂O:

Bactotrypton (Fluka, Buchs, Schweiz)	16.0 g
Bacto yeast extract (Fluka, Buchs, Schweiz)	10.0 g
NaCl (Applichem, Darmstadt, Deutschland)	5.0 g

The DYT media was transferred to a shaking flask and autoclaved.

LB agar plates:

LB agar (Invitrogen, Karlsruhe, Germany)	5.0 g
LB Broth Base (Invitrogen, Karlsruhe, Germany)	10.0 g

500 ml ddH₂O were added and the bottle was autoclaved. After cooling the agar to 50 °C the plates were poured.

2.1.4 Buffers and solutions

Nystatin:

CH ₃ COONH ₄	115.62 g
dissolved in 200 ml ddH ₂ O	
96 % ethanol	200.00 ml
Nystatin	4.00 g

Nystatin was dissolved by adding rotationally the ammonium acetate dissolved in water and the ethanol to a final volume of 400 ml and heating to 55 °C. The nystatin solution was then filtered (0.22 µm) and stored at - 20 °C.

Cholesterol:

1 g Cholesterol was dissolved in 200 ml ethanol (5 mg/ml).

Potassium phosphate buffer (1M, pH 6.0):

KH ₂ PO ₄	108.53 g
K ₂ HPO ₄ * 3 H ₂ O	46.22 g

The compounds were dissolved in 1000 ml ddH₂O and pH was tested before autoclaving.

Potassium citrate (1M, pH 6.0):

Citric acid monohydrate	20.0 g
Tri-potassium citrate monohydrate	293.5 g

The compounds were dissolved in 1000 ml ddH₂O and pH was tested before autoclaving.

Trace metals solution:

disodium EDTA	1.86 g
FeSO ₄ * 7 H ₂ O	0.69 g
MnCl ₂ * 4 H ₂ O	0.20 g
ZnSO ₄ * 7 H ₂ O	0.29 g
CuSO ₄ * 5 H ₂ O	0.025 g

ddH₂O was added to 1000 ml; trace metals solution was sterilized by autoclaving and stored in the dark.

S-basal:

NaCl	5.85 g
K ₂ HPO ₄ * 3 H ₂ O	1.00 g
KH ₂ PO ₄	6.00 g

ddH₂O was added to a final volume of 1000 ml, the pH was set to 6.0 and the buffer was autoclaved before use.

S-medium:

S-basal	1000 ml
Cholesterol (5 mg/ml in ethanol)	1.0 ml
1 M potassium citrate (pH 6)	10.0 ml
Trace metals solution	10.0 ml
1 M CaCl ₂	3.0 ml
1 M MgSO ₄	3.0 ml

IBc:

0.1 M Tris–MOPS	10 ml
0.1 M EGTA/Tris	1 ml
1 M sucrose	20 ml

The volume was filled to 100 ml with ddH₂O, and pH was adjusted to 7.4.

EBc:

1 M KCl	12.5 ml
1 M Tris/MOPS	1.0 ml
0.1 M EGTA/Tris	0.1 ml
1 M Pi	0.1 ml

PH was adjusted to 7.4, and volume was filled to 100 ml with ddH₂O.

2.2 Experimental procedures

2.2.1 *Caenorhabditis elegans* strains and maintenance

All *C. elegans* strains used in this work (wild type Bristol N2; RB754 [*aak-2(ok524)*]; KU25 [*pmk-1(km25)*]; EU31 [*skn-1(zu135)*]; EU1 [*skn-1(zu67)*], LG 357 [*skn-1(zu135)* IV/nT1[*qls51*] (IV;V); *gels10*] (Bishop and Guarente 2007); CF1038 [*daf-16(mu86)*]; PS3551 [*hsf-1(sy441)*]; VC276 [*aip-1(ok537)*]; RB925 [*ire-1(ok799)*]; RB545 [*pek-1(ok275)*]; RB790 [*atf-5(ok576)*]; RB772 [*atf-6(ok551)*]; SJ17 [*xbp-1(zc12)*]; VC1099 [*hsp-4(gk514)*] (Table 1) were provided by Caenorhabditis Genetics Center (University of Minnesota), which is supported by the NIH NCRR.

The temperature-inducible transgenic strain CI 4176 [*smg-1(cc546)* I; *dvIs27 X*] and its control strain CI 802 [*smg-1(cc546)* I; *rol-6(su1006)* II] as first described by Link (Link 1995) were also obtained by the Caenorhabditis Genetics Center (University of Minnesota) and propagated at 16 °C. The expression of muscle-specific A β ₄₂ depends on raising the temperature to 25 °C (Link et al. 2003).

The nematodes were grown on NGM agar plates at 20 °C and maintained as described (Brenner 1974; Zarse et al. 2007).

A stereo microscope (Olympus, Hamburg, Germany) was used for observation, counting and screening of the nematodes.

2.2.2 *Escherichia coli*

The *E. coli* strains OP50 and the streptomycin-resistant OP50i were obtained from Caenorhabditis Genetics Center (University of Minnesota). The bacteria were cultured on LB agar plates at 4 °C. For feeding the worms, DYT media was inoculated with the bacteria for 14 h at 37 °C. Unless otherwise stated the bacteria were then concentrated two fold by centrifuging and discarding half of the supernatant. In a few assays the bacteria were heat-inactivated by shaking them in the incubator at 65 °C for 45 min or heating them in water bath for 30 min at 65 °C. For heat inactivation the complete DYT media was discarded after centrifugation and replaced by a mixture of s-basal with 1 M MgSO₄ and 5 mg/ml cholesterol to a 20 fold concentration.

2.2.3 Compound treatment

Plates containing experimental treatments were prepared from the same batch of NGM agar as the control plates (containing 0.1 % DMSO (Applichem, Darmstadt, Germany) when chemical was dissolved in DMSO) except that the respective chemical was added (Schulz et al. 2007). After the plates were poured, they were sealed and stored at 4 °C. Freshly prepared *E.coli* OP50 were spotted on plates and allowed to dry and grow overnight (Mattison et al. 2012). Incubations with compounds started 64 h after synchronisation of the population, by washing the synchronised, young adult worms and transferring them to the respective treatment plates using s-buffer.

Butylated hydroxyanisole (BHA) was dissolved in DMSO (10 mM stock solution). Four generations of nematodes (wild type Bristol N2) were propagated on agar plates containing 10 µM BHA before initiation of experiments (Zarse et al. 2012).

For punicalagin (Phytolab, Vestenbergsgreuth, Germany) supplementation experiments, the polyphenol was added to autoclaved agar at 50 °C as a 1 mM stock solution in DMSO to obtain a final concentration of 1 µM.

RO 90-7501 was also added as a 1 mM stock solution in DMSO with a final concentration of 1 µM.

2.2.4 Treatment of *C. elegans*

For all the performed assays the nematodes were treated as mapped in Figure 7, except for the lifespan analyses and the paralysis assay.

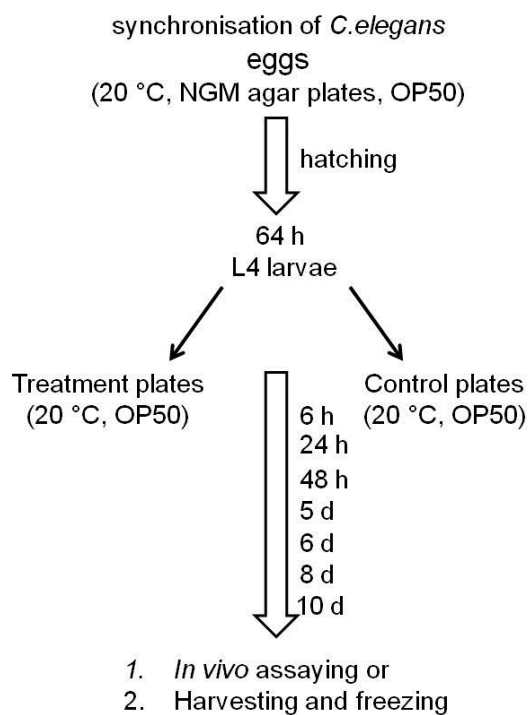


Figure 7: Procedure for the treatment of *C. elegans*

Nematodes were synchronised. The observed eggs were allowed to hatch. Young adult worms were washed off the plates and distributed to treatment plates containing the respective compound and to control plates containing the solvent. After reaching the desired time points the nematodes were used for *in vivo* assays or were frozen in liquid nitrogen.

Treatment plates contained the respective compound. Control plates contained 0.1 % DMSO when chemical was dissolved in DMSO or ddH₂O when chemical was dissolved in water.

2.2.5 Synchronisation

A non-bleaching synchronisation was performed by isolating eggs from a mixed population. The worms were washed off the plates with sterile ddH₂O and were discarded. The bacterial lawn including the eggs was then scraped off the plate and transferred into a conical centrifuge tube. After centrifugation (1300 g for 1 min) the eggs formed a pellet following gravity-based separation (Macario and Conway de Macario 2002) and the bacterial suspension was removed. The eggs were washed another two times with sterile ddH₂O to remove all the

bacteria. The remaining eggs were transferred to NGM agar plates and incubated at 20 °C. The following day, all abnormal worms were removed from the plates.

2.2.6 Lifespan analyses

Lifespan was assayed at 20 °C according to standard protocols (Schulz et al. 2007) without the use of 5'-fluorouridine. A non-bleaching synchronisation was performed as described above and was counted as day 0 of lifespan analyses. Approximately 100 to 150 worms of the prefertile young adult stage of the synchronized nematode population were transferred to NGM agar plates spotted with *E. coli* strain OP50 and supplemented with test compounds or control 64 h after synchronisation. Experiments were conducted in triplicates. Nematodes were manually transferred to fresh plates every day during the progeny production period and every second day thereafter. Plates were monitored for dead animals. Nematodes showing no reaction to gentle stimulation were scored as dead. Animals that crawled off the plates or displayed unnatural death (particularly due to internal hatching) were censored (Schulz et al. 2007). At day 12 the nematodes were transferred to NGM agar plates containing streptomycin (200 µg/ml) and covered with the streptomycin-resistant *E. coli* strain OP50i.

2.2.7 Locomotion assay

Single-worm movements within a liquid system were recorded using a digital CCD camera (Moticam 2300, Motic, St. Ingbert, Germany) coupled microscope (SMZ 168, Motic, St. Ingbert, Germany) and analysed with DanioTrack (Loligo Systems, Tjele, Denmark). Worms were treated for individual time points as mapped in Figure 7. Ten worms were transferred from the plates into s-buffer. A single worm was transferred to a glass slide with 1 µl s-buffer and 20 seconds of video were immediately recorded. During the subsequent video analyses, the DanioTrack software subtracted the background and determined the centre of gravity of all object pixels in contrast with the background. Finally, the distance that the worm moved from the centre of gravity was tracked and calculated. Thus, this locomotion assay can be considered as quantitative analyses of the maximum movement capacity of a single worm.

2.2.8 Pumping rate

Worms were treated for individual time periods as mapped in Figure 7. After reaching the desired time points, the pumping rate of the nematodes was measured by transferring 10 to 15 worms to a glass slide covered with a 5 % agarose gel and 5 μ l of bacteria (heat-inactivated OP50). Another agarose slide was placed on top to fix the worms and prevent rapid movement. Pharyngeal pumping was determined by counting singular movements of the pharynx. Videos were recorded (Jenoptik ProgRes CT, Jena, Germany) for 30 s and pumps were counted by watching the video in slow motion.

2.2.9 Paraquat stress resistance assay

Stress resistance to a lethal oxidative stress derived by paraquat was determined as described (Schulz et al. 2007) with minor modifications. For 6 days, synchronised nematodes were maintained on NGM agar plates containing the compound or 0.1 % DMSO as control and 100 μ g/ml ampicillin and being spotted with heat-inactivated OP50 (45 min at 65°C in the incubator). To prevent mixing of generations, nematodes were transferred to new plates every day by washing with s-basal followed by gravity-based separation. Six-day-old (after L4) nematodes were manually transferred to fresh plates containing 10 mM paraquat (Acros Organics, Geel, Belgium) and spotted with heat-inactivated OP50. The survival rate was determined daily by counting dead and censored worms as described for the lifespan analyses.

2.2.10 Thermotolerance stress resistance assay

The “SYTOX Green assay” was used to determine the survival of worms at a lethal temperature of 37 °C. The method was developed by Gill et al. (Gill et al. 2003) and modified by Kampkötter et al. (Kampkötter et al. 2007). After treating the nematodes for 6 days (Figure 7) the worms were washed off the plates with s-medium. After washing for 90 min in s-medium, the worms were individually transferred with 1 μ l of s-medium to the wells of a 384-well microtitre plate (Greiner Bio-One, Frickenhausen, Germany) containing 4,5 μ l s-medium, 4,5 μ l SYTOX Green (final concentration 1 μ M) (Molecular Probes Inc., Leiden, Netherlands) and 10 μ l of a bacterial suspension (heat-inactivated, 10^7 bacteria/ml). The microtitre plate was sealed with backSeal-96/384 (Perkin Elmer Wellesley, USA). The fluorescence intensity was determined at 37 °C

over 20 h. Because of thermal stress the worms die and the dye can enter the impaired cells, bind to the DNA and exert a bright fluorescence (Brown and Naidoo 2012). The fluorescence (excitation 485 nm, emission 535 nm) was measured in intervals through the transparent bottom of the plate and fluorescence curves were calculated for each well. The cut off value was determined by multiplying the first measurements by a factor of three (Brown and Naidoo 2012). The time when the fluorescence raised above the cut off value was defined as the point of death for this worm. The survival curves were calculated from the individual time points of death.

2.2.11 Determination of ATP

A worm-lysate-based method (Yang et al. 2002) was used to determine the ATP content of *C. elegans*. Wild type worms were harvested and immediately shock frozen in liquid nitrogen. The frozen worm pellet was ground and transferred to a cooled microfuge tube. Pre-heated 4 M guanidinium-HCL (Carl Roth GmbH & Co KG, Karlsruhe, Germany) was added to the frozen worm powder to inactivate the ATPase and was boiled for 15 min at 99 °C. After centrifuging (4 °C, 13200 g, 30 min) and transferring the supernatant to a new centrifuge tube, the ATP content was determined by using a commercially available kit (CellTiter-Glo; Promega, Fitchburg, WI, USA). The boiled samples were diluted 1 to 100 with ddH₂O depending on the size of the worm pellet. One-hundred µl of the dilution was pipetted in to a white 96-well LIA microtitre plate (Greiner Bio-One, Frickenhausen, Germany) and 50 µl CellTiterGlo were added. After 40 min of incubation, the luminescence (arising from the reaction shown in Figure 8) was measured in a microplate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). For normalisation the luminescence signal was related to the protein content, which was determined with BCA.

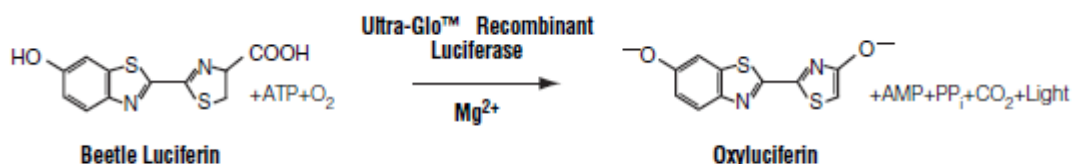


Figure 8: The luciferase reaction

In the presence of ATP, luciferin is oxidized to oxyluciferin. This reaction is catalysed by luciferase.

(Promega CellTiter-Glo Luminescent Cell Viability Assay Technical Bulletin)

2.2.12 Respiration assay

Respiration was quantified using a DW1/AD Clark-type electrode (Hansatech, King's Lynn, England) as described (Schulz et al. 2007). Worms were treated for individual time periods as mapped in Figure 7. After reaching the desired time point the worms were washed off the plates with s-buffer and transferred into a calibrated DW1 chamber. Oxygen consumption was monitored for at least 10 min. The worms were then carefully removed from the chamber into cooled microfuge tubes and protein content was determined with Bradford. The nematodes were sonicated 3 times (intensity 50 %, 10 s); centrifuged 15 min at 12000 g and the supernatant was used for protein determination with Bradford. Oxygen consumption was calculated by dividing the oxygen consumption rate by the protein content of each sample.

2.2.13 Mitochondrial ROS levels

MitoTracker Red CM-H₂XRos (Invitrogen, Carlsbad, CA, USA) is used to determine radical formation in the mitochondria. This reduced probe does not fluorescence until it enters live cells, where it is oxidised to the corresponding fluorescent mitochondrion-selective probe and then sequestered in the mitochondria (Invitrogen MitoTracker Red CM-H₂XRos Technical Bulletin).

After nematodes were treated for individual time periods as mapped in Figure 7, they were washed off the plates with s-buffer and allowed to settle for separation of the offspring. Worms were washed another two times and transferred to freshly prepared MitoTracker plates. These plates were spotted with 500 µl of heat-inactivated OP50 (30 min, 65 °C in water bath) and 100 µl MitoTracker Red CM-H₂XRos solution in DMSO (100 µM) and allowed to dry for 20 min. After incubating for 2 h the worms were washed off the plates and then washed

another two times. To remove excess dye from the gut the nematodes were transferred to NGM agar plates spotted with OP50 and containing the compounds or control and rested there for about 1 h at 20 °C. The worms were washed off the plates. Aliquots of 100 µl worm suspension were pipetted into eight wells each treatment group of a 96-well FLUOTRAC™ plate (Greiner Bio-One, Frickenhausen, Germany). Fluorescence intensity was measured in a micro plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany) using well-scanning mode (excitation: 570 nm, emission: 610 nm). To normalise fluorescence signal, the worms were removed from the wells, washed with s-buffer, sonicated (4 times, 70 %), centrifuged (12000 g, 15 min) and the supernatant was used for protein determination with Bradford.

2.2.14 Amplex Red-based determination of hydrogen peroxide levels

The nematodes were incubated with the compounds or the control for individual time periods (Figure 7). The amount of hydrogen peroxide produced was measured by washing the worms off the plates with a 0.05 M sodium phosphate buffer (pH 7.4) and transferring them to an upright plexiglas cylinder (1.5 ml volume) with continuous stirring at low speed. One-and-a-half µl Amplex Red dye (Invitrogen, Karlsruhe, Germany) dissolved in DMSO was added to the worm suspension to give a final concentration of 1 µM. First fluorescence was measured without horseradish peroxidase in the presence of Amplex Red to detect possible unspecific increase in fluorescence. Afterwards, 15 µl of horseradish peroxidase (diluted in 0.05 M sodium phosphate buffer) was added (end concentration 0.01 U/ml). Changes in fluorescence were recorded up to 20 min with a fluorescence detector (LF402 ProLine, IOM, Berlin, Germany) at excitation and emission wavelengths of 571 nm and 585 nm. Horseradish peroxidase catalyses the reaction of hydrogen peroxide with Amplex Red to produce highly fluorescent resorufin (Invitrogen Amplex Red Technical Bulletin). Immediately afterwards worms were collected for protein determination with Bradford to normalise fluorescence values.

2.2.15 Antioxidant enzyme activities

Antioxidant enzyme activities in nematodes were assayed by using commercially available kits for catalase (CTL) (Calbiochem, Darmstadt, Germany) and superoxide dismutase (SOD) (Sigma-Aldrich, Munich, Germany). For each time point and treatment, three independent worm samples were collected, frozen in liquid nitrogen and stored at -80°C . After grinding, sonication and centrifugation an aliquot of the supernatant was used for the measurement. The remaining supernatant was used for protein determination with Bradford. After calculating the protein content the SOD samples were diluted to give a final protein concentration of $0.1\text{ }\mu\text{g}/\mu\text{l}$. SOD activity was determined according to the manufacturer's protocol. WST-1 reacts with superoxide anion to produce a water-soluble formazan dye. The rate of this reduction is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD (Figure 9). The absorption of WST-1 formazan was measured at 440 nm in a micro plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). Since the SOD activity is an inhibition activity, the decrease in the colour development was measured (Sigma-Aldrich SOD Assay Kit User Protocol).

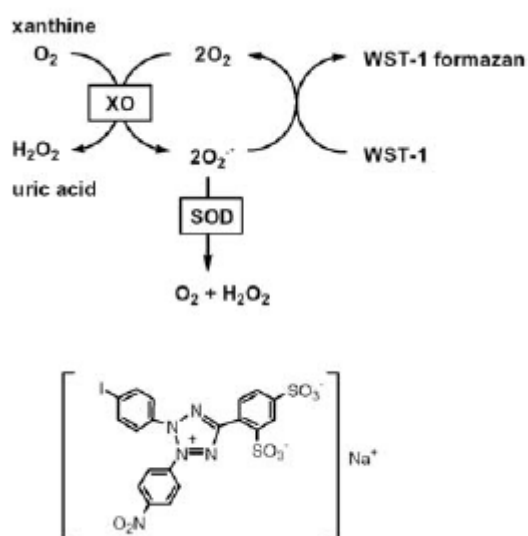


Figure 9: Principle of the SOD determination

(Sigma-Aldrich SOD Assay Kit User Protocol)

Catalase activity was determined similarly to SOD, except that the CTL samples were diluted to give a final protein concentration of $1\text{ }\mu\text{g}/\mu\text{l}$. The assay was performed according to the manufacturer's protocol. The method is based on

the reaction of catalase with methanol in the presence of hydrogen peroxide. Formaldehyde is produced and can be photometrically measured using Purbald as a chromogen. Purbald changes from colourless to a purple colour after reacting with aldehydes (Calbiochem Catalase Assay Kit User Protocol).

2.2.16 Glucose oxidation assay

Animals were treated for 6 h on NGM agar plates with the compound as described above (Figure 7). After washing three times with s-buffer worms were collected and each pellet was resuspended in 700 μ l incubation buffer containing s-buffer with 0.5 mM non-radioactive glucose and 17 μ M labeled [14 C] D-glucose (5 μ Ci/ml) (GE Healthcare, Buckinghamshire, UK). The suspension was transferred to a 4 cm Petri dish, which was placed in a 10 cm Petri dish (without a lid) together with a second 4 cm Petri dish (without a lid) containing 600 μ l of 0.1 M KOH solution to trap CO₂ (Ristow et al. 2000). The 10 cm dish was sealed with parafilm. The nematodes rested in the incubation buffer for about 3 h and afterwards 500 μ l of the responding KOH solution from the sealed 10 cm Petri dish was transferred into 4.5 ml scintillation liquid for radioactivity measurement in a Beckman counting machine (Beckman LS 6000, Global Medical Instrumentation, Inc.) to quantify the amount of trapped 14 CO₂. The worms were transferred from the Petri dishes to microfuge tubes for a protein determination with Bradford.

2.2.17 Fat content

For fat content determination, the nematodes were treated with the respective compounds for individual time periods as described before (Figure 7). After reaching the desired time point the nematodes were washed off the plates with ice cold s-buffer and centrifuged (200 g, 1 min, 4 °C). After washing the worms twice with ice cold s-buffer the worms were transferred into ice cooled reaction tubes and centrifuged at 3000 g for 1 min at 4 °C. The supernatant was completely removed and the remaining worm pellet was shock frozen in liquid nitrogen and stored at -80 °C. Before determining fat content, the worm pellets were ground in a nitrogen-chilled mortar with 200 μ l s- buffer. The frozen material was transferred to a new reaction tube and placed on ice (400 to 800 μ l s-buffer were added after grinding). After sonication 200 μ l of the supernatant was used for a protein determination with BCA after centrifugation (15 min,

12000 g, 4 °C). The remaining solution was slowly heated to 80 °C in a thermomixer (beginning with 25 °C and heated to 80 °C (approx. 10 min). The sample was then shaken for 5 min at the highest speed. The solution was slowly cooled to ambient temperature. The heating procedure was repeated one more time to solubilise all the triglycerides. To remove any insoluble material a centrifugation step (5 min, 8000 g) followed. The supernatant was transferred into a new reaction tube.

Glycerol determination was carried out in 96-well microtitre plates. Glycerol standards needed to be prepared in the concentrations 0; 0,1; 0,25; 0,5; 1; 1,5; 2 µg/µl; and 1 µl was pipetted into four wells each. Furthermore 10 µl of the samples were pipetted into four wells for each treatment. Two-hundred µl glycerol determination assay reagent from Triglyceride Assay Kit (Roche Diagnostics, Mannheim, Germany) was added to standards and samples. Triglycerides are hydrolysed to glycerol by the enzyme lipoprotein lipase. This is followed by an oxidation to dihydroxyacetone phosphate and hydrogen peroxide. The resulting hydrogen peroxide reacts with 4-aminophenazone and 4-chlorophenol to a red dye, which is catalysed by the enzyme peroxidase (Roche Diagnostics Triglycerides GPO-PAP Determination Assay Kit User Protocol). After 15 min of incubation at ambient temperature, the absorbance was measured at 544 nm in a micro plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). The colour intensity of the red dye is directly proportional to the triglyceride content.

2.2.18 Paralysis assay

A transgenic *C. elegans* strain CI 4176 [*smg-1(cc546)* I; *dvls27* X] and its control CI 802 [*smg-1(cc546)* I; *rol-6(su1006)* II] were used for the paralysis assay. This model carries an A β ₄₂ transgene with temperature-inducible muscle expression resulting in a paralytic phenotype after temperature up-shift (Link et al. 2003). Treatments that counter the formation of A β ₄₂ toxicity alter the paralysis rate of the transgenic nematodes. The assay was performed as described (Dostal and Link 2010, Diomedea et al. 2010) with minor changes.

Worms were maintained at 16 °C on NGM agar plates spotted with OP50 and egg-synchronised onto NGM agar plates containing either the control or the compound. The plates were then returned to 16 °C and the nematodes were

allowed to hatch. Fifty-four h after the synchronization, the temperature was shifted to 25 °C. The up-shift of temperature must occur before the worms reach the mid L4 larvae stage because the expression of the *myo-3* promoter is significantly reduced in adult worms. Twelve to sixteen h later 100 worms were transferred to new NGM agar plates containing the compound or control and spotted with OP50. Twenty-eight h after the up-shift of temperature the nematodes were counted every 2 h for paralysis (Figure 10).

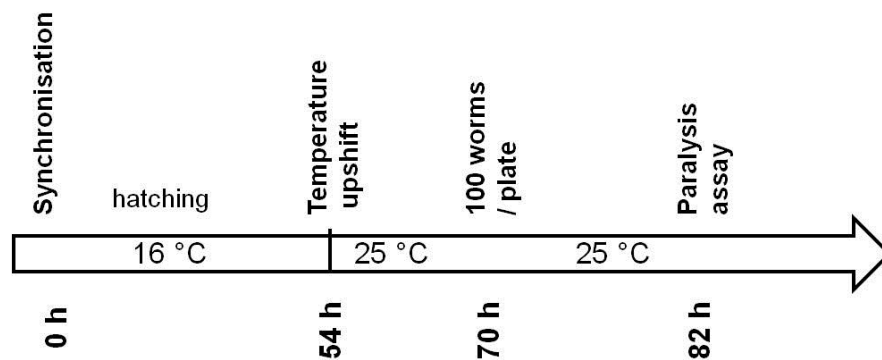


Figure 10: Timetable for the Paralysis assay

Paralysis does not always occur over the whole body length; the head region is the last part of the worm to cease moving. Worms that can still to move their heads form “halos” of cleared bacteria and were also counted as paralysed. Worms that were prodded with the worm picker and did not show a full body wave were also counted as paralysed. The nematodes of the control strain CI 802 [*smg-1(cc546)* I; *rol-6(su1006)* II] were prepared and handled the same way but did not undergo paralysis after the temperature up-shift to 25 °C.

2.2.19 Isolation of mitochondria from rat liver and respiration measurement

The isolation of mitochondria of rat liver was performed as described for mouse liver (Frezza et al. 2007). The rat was starved overnight before the experiment, killed and the liver was excised. The liver was provided by the institute for laboratory animal science and animal protection (University Medical Centre, Jena, Germany). The liver was rinsed four or five times free of blood with ice-cold IB_c buffer and minced to small pieces.

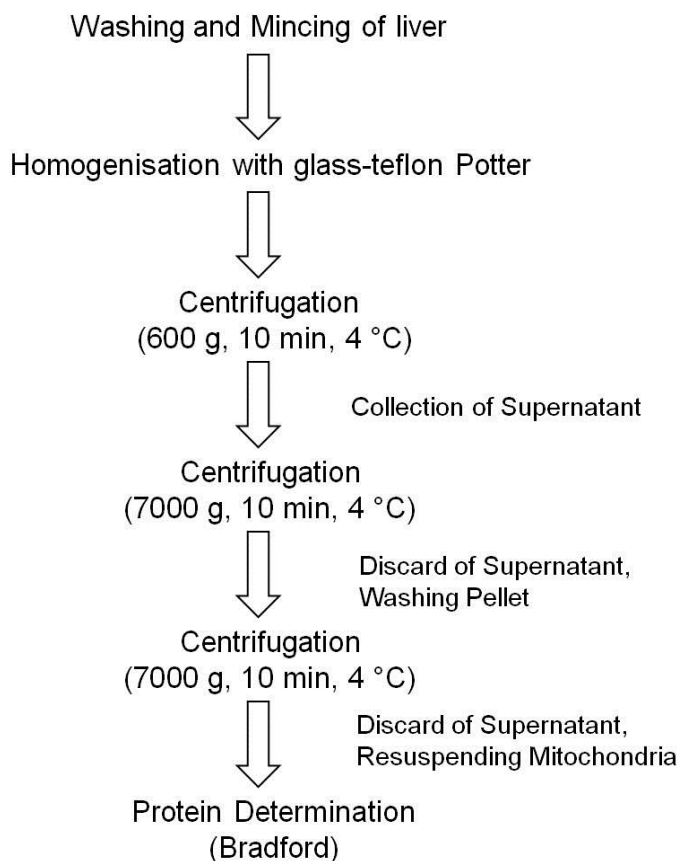


Figure 11: Procedure for the isolation of mitochondria from liver

based on (Frezza et al. 2007)

A suspension of minced liver and 5 ml of IB_c was then homogenised with a loose glass-teflon potter five to six times per hand and afterwards with a tight glass-teflon potter. The homogenate was transferred to a 50 ml conical centrifuge tube and centrifuged at 600 g for 10 min at 4 °C. The remaining steps of the procedure are mapped in Figure 11. Oxygen consumption was quantified using a calibrated DW1/AD Clark-type electrode (Hansatech, King's Lynn, England). One ml of freshly prepared EB_c buffer was transferred into the chamber, 300 µg of the mitochondria were added, and oxygen consumption was monitored at 30 °C (state 1). After monitoring for 2 min, the substrates for complex I, glutamate (5 mM) and malate (2.5 mM) were added. After recording the oxygen consumption for another few minutes, ADP (100 µM) was added. Increased consumption of oxygen was observed (state 3). Oxygen consumption was monitored until respiration slowed down and reached the values before the addition of ADP (state 4). For uncoupling the mitochondria FCCP (100 nM) was added and the respiration reached levels higher than state 3.

To analyse the respiration changes of the mitochondria with application of different substances the compounds were added after state 4, followed by an additional ADP so that the respiration was recorded in the following order: state 3 and state 4 of mitochondria and state 3 and state 4 of mitochondria influenced by the compounds. For comparing the effects of the substances to the control a quotient of state 3 (compound or control) to state 4 (mitochondria) was calculated.

2.2.20 Complex I activity assay with mitochondria from mouse liver

The liver was provided by the institute for laboratory animal science and animal protection (University Medical Centre, Jena, Germany).

Mouse mitochondria were isolated as described above for rat liver, dissolved in Tris (10 mmol/l, pH 7.6) and stored at - 80 °C. The assay was performed as described by Janssen et al. (Janssen et al. 2007).

Complex I oxidises NADH and the electrons produced reduce decylubiquinone which delivers the electrons to DCIP. The reduction of DCIP can be measured spectrophotometrically at 600 nm (Janssen et al. 2007). The assay is carried out in a 24-well plate (Biochrom AG, Berlin, Germany). In each well a mixture of 899 µl potassium phosphate buffer (25 mmol/l pH 7.4), 6 µl DCIP (60 µmol/l), 4 µl decylubiquinone (17.5 mmol/l in DMSO), 1 µl antimycin A (1 mmol/l in DMSO) and 50 µl BSA (70 g/l in 5 mmol/l potassium phosphate) was pipetted, and 20 µl of mitochondria in Tris (10 mmol/l, pH 7.6) were added. After a preincubation of 3 min, 20 µl NADH (10 mmol/l) was added and lightly shaken for a few seconds. The absorption was measured in 30 s intervals for 4 min at 20 °C at 600 nm. Afterwards, 1 µl rotenone (1 mmol/l in DMSO) as the positive control or 1 µl of the respective compounds (10 mmol/l in DMSO) or the control (DMSO) was added. A short shaking step was followed by another absorption measurement (30 s intervals, 10 min, 20 °C).

2.2.21 Protein determination

Protein content in nematodes was determined by standard methods. The Bradford protein assay was performed as first described by M.M. Bradford (1976). It is a colorimetric protein assay where the dye coomassie brilliant blue is converted from the red form to the bluer one to bind the protein being assayed. The bound form of the dye has an absorbance of 595 nm, whereas the unbound form has its maximum at 465 nm. The increase of the absorbance at 595 nm is proportional to the amount of proteins (Bio-Rad, Hercules, California, Protein Assay Protocol).

Because some detergents seemed to interfere with the Bradford dye another standard assay was used for protein determination, the BCA assay. The colour changes of the bicinchoninic acid (BCA, Thermo Scientific, Germany) from green to purple are proportional to the protein content. The absorbance of the purple coloured product was measured at 560 nm in a micro plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany). This assay was performed as described by Smith, Krohn et al. (1985). The protein samples and standards were pipetted into a 96 well plate and 100 μ l of BCA reagent was added to each well. After a short mixing step the plate was incubated for 30 min at 37 °C to increase the sensitivity of the assay. The final absorbance was measured at 560 nm.

2.2.22 Statistical analyses

Data are expressed as means \pm SD unless otherwise indicated. After testing for equal distribution of the data and equal variances within the data set, a Student's t-test (unpaired, two-tailed) was used to compare all data except for lifespan and stress resistance assays. For comparing significant distributions between different groups in the lifespan and stress resistance assays, statistical calculations were performed using JMP software version 9.0 (SAS Institute Inc., Cary, NC, USA) applying the log-rank test. All other calculations were performed using Excel 2007 (Microsoft, Albuquerque, NM, USA). A p-value below 0.05 was considered statistically significant.

3. Results

3.1 Modulation of life expectancy of *C. elegans* by punicalagin and RO 90-7501

Different concentrations of punicalagin and RO 90-7501 were used to analyse the lifespan-modulating effects on *C. elegans*. Nematodes were exposed to punicalagin or RO 90-7501 from the L4 larval stage to death, and survival rates were scored. For both compounds, a final concentration of 1 μ M in the agar was the most effective.

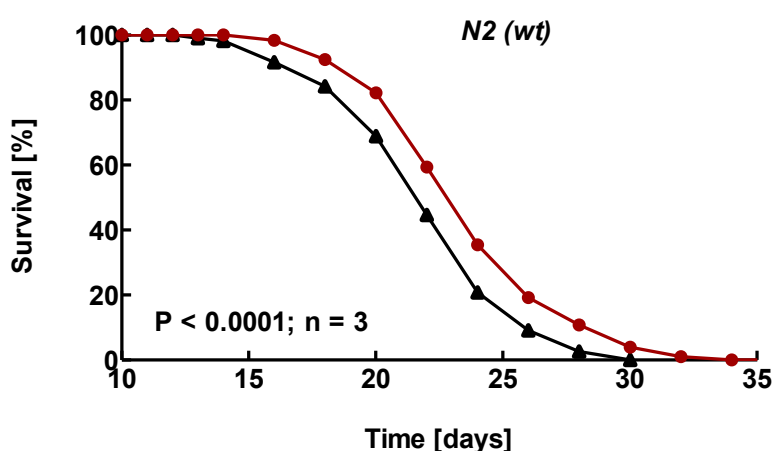


Figure 12: Punicalagin extends lifespan of *C. elegans*

Lifespan analyses of N2 wild type nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

N2 wild type nematodes were exposed to 1 μ M punicalagin from L4 larval stage to death and had significantly higher ($p < 0.0001$) survival rates than control worms treated with 0.1 % DMSO (Figure 12), which was represented by a shift of the survival curve to the right. Punicalagin-treated worms had a mean lifespan of 24.07 ± 0.3 d and a maximum lifespan of 26.7 ± 0.7 d, whereas control nematodes showed a mean lifespan of 22.41 ± 0.2 d and a maximum lifespan of 24.7 ± 0.7 d. The mean lifespan was extended by 1.66 d (7.41 %) and the maximum lifespan by 2 d (8.10 %).

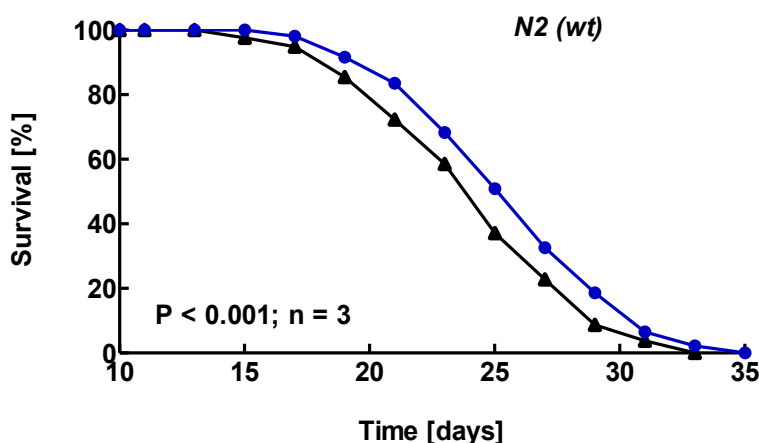


Figure 13: RO 90-7501 extends lifespan of *C. elegans*

Lifespan analyses of N2 wild type nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

Exposure to 1 μ M Ro 90-7501 significantly ($p < 0.001$) extended the lifespan (Figure 13) of wild type nematodes in a concentration of 1 μ M in the agar in comparison to untreated nematodes, which is represented by a shift of the survival curve to the right. The RO 90-7501-treated worms had a mean lifespan of 26.18 ± 0.2 d and a maximum lifespan of 29.0 ± 0.0 d, whereas control nematodes had a mean lifespan of 24.66 ± 0.3 d and a maximum lifespan of 27.7 ± 0.7 d. The mean lifespan was extended by 1.52 d (6.16 %) and the maximum lifespan by 1.3 d (4.69 %).

3.2 The hydrolysis product of punicalagin does not influence life expectancy of *C. elegans*

The ellagitannin punicalagin spontaneously hydrolyses into smaller phenolic compounds such as ellagic acid (Larrosa et al. 2006). Therefore, the modulating activities of ellagic acid on lifespan of *C. elegans* wild type nematodes at 20 °C were tested. The dissolved compound was applied to the agar in a final concentration of 1 μ M.

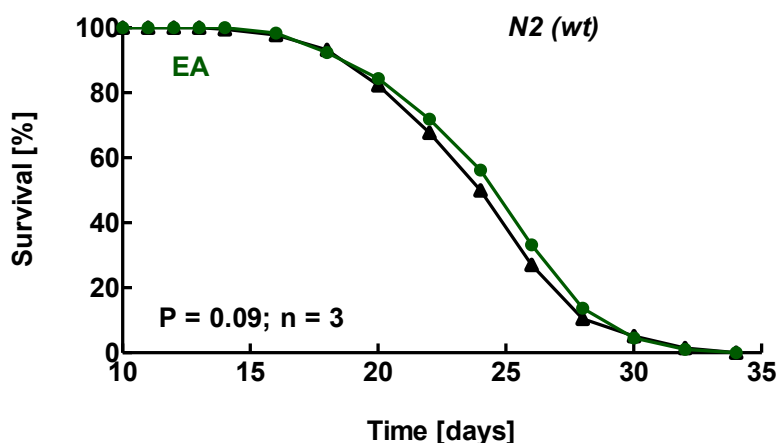


Figure 14: Ellagic acid has no influence on lifespan of *C. elegans*

Lifespan analyses of *N2* wild type nematodes in the presence (green) or absence (black) of 1 μ M ellagic acid (EA). Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

In this work no significant lifespan-extending effect could be determined for 1 μ M ellagic acid in the agar (Figure 14). The survival rates of the nematodes exposed to ellagic acid were similar to those of the untreated worms.

3.3 Punicalagin and RO 90-7501 increase locomotion of *C. elegans*

Because of the lifespan-enhancing effects of punicalagin and RO 90-7501, one important parameter for the quality of life, the mobility, was monitored. Locomotion is a parameter that represents the health and fitness of the nematodes. *Caenorhabditis elegans* wild type nematodes were treated with 1 μ M punicalagin from the L4 larval stage to 10 d, and with 1 μ M RO 90-7501 to 8 d, and locomotion was determined by recording the distance covered per second.

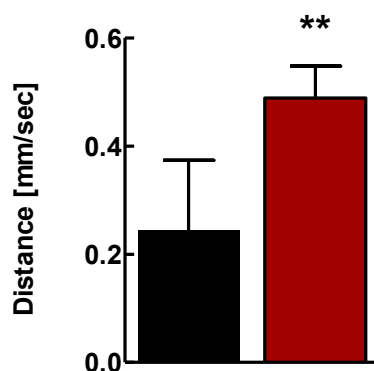


Figure 15: Punicalagin increases locomotion of *C. elegans* after a 10 d treatment

Covered distance (mm/sec) of nematodes (n = 10) after 10 days of treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

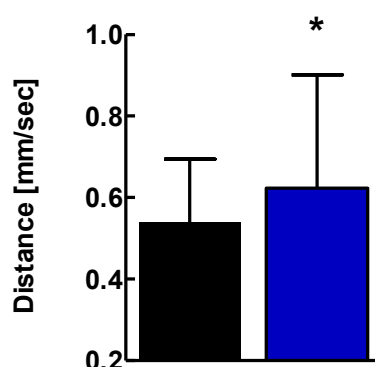


Figure 16: RO 90-7501 increases locomotion of *C. elegans* after an 8 d treatment

Covered distance (mm/sec) of nematodes (n = 10) after 8 days of treatment with 1 μ M RO 90-7501 (blue) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

Both punicalagin (p < 0.01) (Figure 15) and RO 90-7501 (p < 0.05) (Figure 16) were capable to significantly increase the locomotion of wild type nematodes after exposure for 10 d (punicalagin) or 8 d (RO 90-7501) in comparison to untreated worms. The nematodes were able to cover a longer distance in 1 s than the control worms, implying a better health and fitness of the treated nematodes.

3.4 Ro 90-7501 induces stress resistance against oxidative and thermal stress

Lifespan extension is correlated with stress resistance against oxidative and/or thermal stress (Macario and Conway de Macario 2002). In this work we tested whether the lifespan extension mediated by RO 90-7501 is also correlated with an increase in stress resistance. First, the survival of nematodes under oxidative stress generated by exposure to paraquat was determined. The intracellular toxicity of paraquat is caused by a massive formation of superoxide radicals (Bagley et al. 1986). Wild type nematodes were preincubated with 1 μ M RO 90-7501 for 6 d starting with L4 and afterwards exposed to 10 mM paraquat dissolved in the agar. Survival rate was determined.

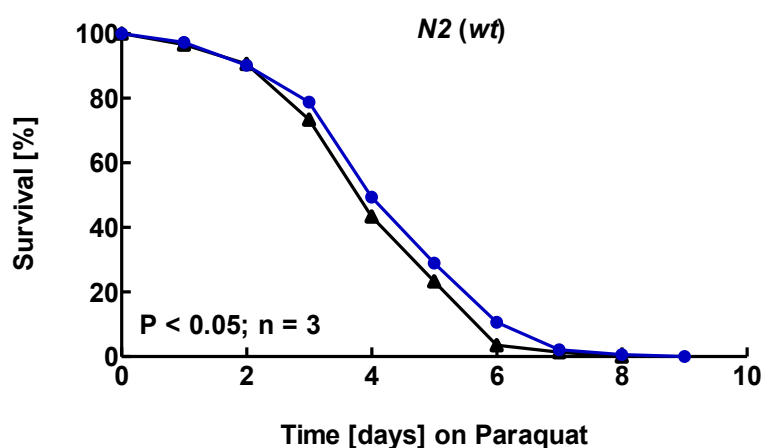


Figure 17: RO 90-7501 increases resistance against oxidative stress in *C. elegans*

C. elegans survival during paraquat exposure following 6 days of RO 90-7501 pretreatment (blue, $n = 218$) vs. control (black, $n = 215$). Values are given as mean out of 3 independent experiments.

After a 6 d incubation with 1 μ M RO 90-7501, the nematodes showed significant ($p < 0.05$) more resistance against oxidative stress generated by paraquat as reflected by an increased survival rate compared to untreated nematodes (Figure 17).

Second, the survival rate of wild type nematodes under thermal stress (37 °C) was determined after a 6 d incubation with 1 μ M RO 90-7501. Acute thermal stress released by the temperature up-shift to 37 °C leads to rapid death of *C. elegans*.

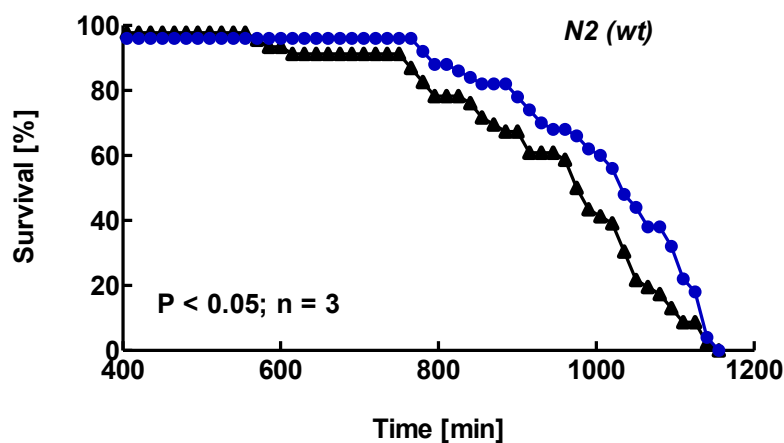


Figure 18: RO 90-7501 increases resistance against thermal stress in *C. elegans*

C. elegans survival during thermal stress (37 °C) following 6 days of Ro 90-7501 pretreatment (blue, n = 50) vs. control (black, n = 46).

The nematodes exposed to 1 μ M RO 90-7501 showed a significantly ($p < 0.05$) higher survival rate than the control nematodes at 37 °C (Figure 18), which is represented by a shift of the survival curve to the right.

3.5 Influence of punicalagin and RO 90-7501 on oxygen consumption

Respiration, an important metabolic parameter representing the amount of oxygen consumed by the nematodes, was investigated for possible modulating effects generated by punicalagin or RO 90-7501. Wild type nematodes were treated with 1 μ M of both compounds for different time periods, and oxygen consumption was determined *in vivo*.

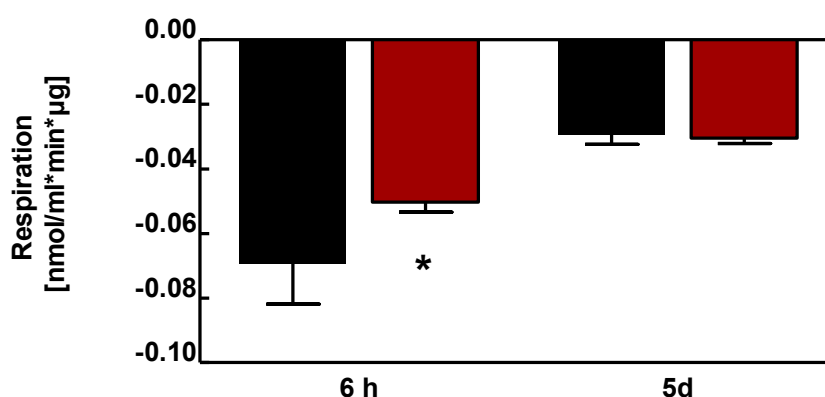


Figure 19: Modulation of oxygen consumption by punicalagin

Oxygen consumption (nmol/ml*min* μ g) of nematodes (n = 5) after a 6h and 5 day treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After 6 h of treatment with 1 μ M punicalagin the nematodes showed a significantly (p < 0.05) reduced respiration rate compared to the control (Figure 19). The short-term inhibition of respiration was amounted to 27.73 %. After 5 d of application the reduction in oxygen consumption was compensated. No significant difference could be determined in comparison to the untreated control group.

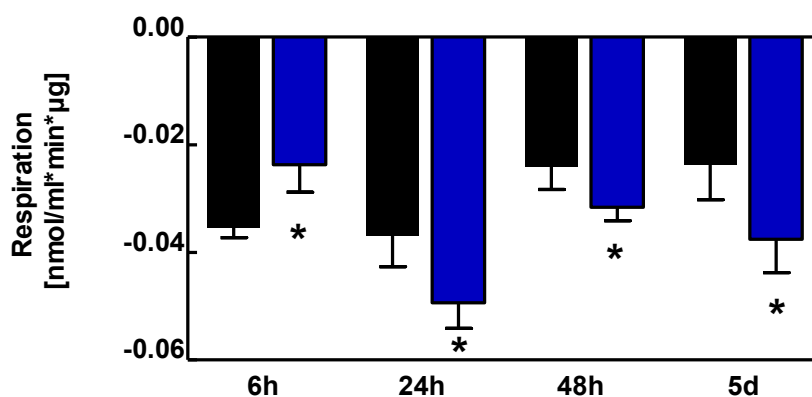


Figure 20: Modulation of oxygen consumption by RO 90-7501

Oxygen consumption (nmol/ml*min*µg) of nematodes (n = 5) after a 6h, 24h, 48h and 5 d treatment with 1 µM RO 90-7501 (blue) or 0.1 % DMSO (black). Values are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After 6 h of incubation with 1 µM RO 90-7501 a significant (p < 0.05) inhibition of oxygen consumption (Figure 20) occurred. The inhibition reached 32.86 %. Twenty-four h after exposure, RO 90-7501 increased mitochondrial oxygen turnover. Nematodes consumed significantly (p < 0.05) more oxygen (33.86 %) than the control. This induction of respiration could also be detected after 48 h (p < 0.05) and 5 d (p < 0.05).

3.6 Complex I activity of isolated mouse mitochondria under the influence of punicalagin and RO 90-7501

Because of the temporary decrease in respiration of *C. elegans* after exposure to 1 µM punicalagin and 1 µM RO 90-7501, both compounds were analysed for their capability to inhibit the activity of complex I of the ETC. Therefore, the activity of complex I was assayed with isolated mouse mitochondria. Besides both compounds in concentrations of 10 µM, rotenone (1 µM) was used as a positive control.

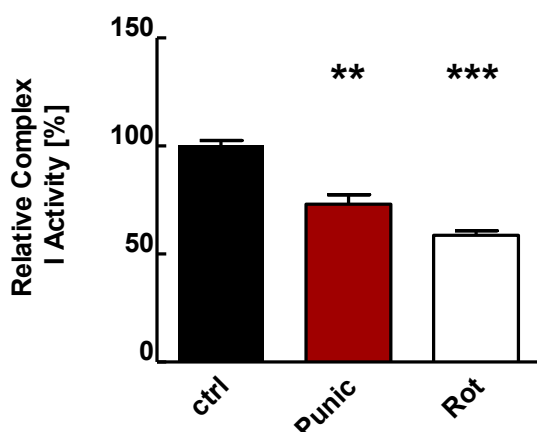


Figure 21: Complex I activity of isolated mouse mitochondria is inhibited by punicalagin

Complex I activity (% control) of isolated mouse liver mitochondria (n = 4) treated with 10 μ M punicalagin (Punic), 0.1 % DMSO (ctrl) or 1 μ M rotenone (Rot). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

Punicalagin (Figure 21) and RO 90-7501 (Figure 22) significantly (p < 0.01) inhibited complex I activity in isolated mouse mitochondria. The inhibition by punicalagin reached 26.97 % compared to untreated control mitochondria. RO 90-7501 reduced the complex I activity by 13.3 %. As expected, rotenone inhibited the complex I activity by 41.34 %.

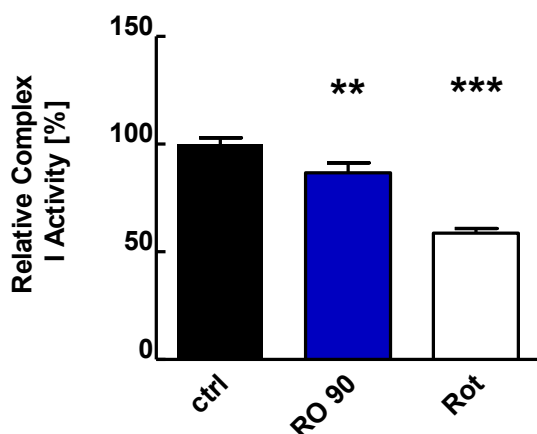


Figure 22: Complex I activity of isolated mouse mitochondria is inhibited by RO 90-7501

Complex I activity (% control) of isolated mouse liver mitochondria (n = 4) treated with 10 μ M RO 90-7501 (RO 90), 0.1 % DMSO (ctrl) or 1 μ M rotenone (Rot). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

3.7 Respiration of isolated rat mitochondria under the influence of punicalagin and RO 90-7501

The complex I activity assay showed an inhibition of complex I activity afforded by punicalagin and RO 90-7501. To verify this, the respiration of isolated rat liver mitochondria was additionally measured. The oxygen consumption was monitored while adding the substrates for complex I, glutamate (5 mM) and malate (2.5 mM). The modulation of respiration rate was analysed with and without application of 10 μ M punicalagin and 10 μ M RO 90-7501. Rotenone was used as positive control because rotenone inhibits complex I of the electron transport chain (Tuohimaa et al. 2004).

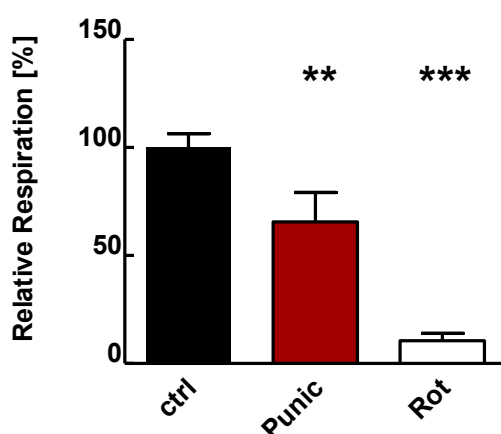


Figure 23: Respiration of isolated rat mitochondria is inhibited by punicalagin

Oxygen consumption (% control) of isolated rat liver mitochondria (n = 5) treated with 10 μ M punicalagin (Punic), 0.1 % DMSO (ctrl) or 1 μ M rotenone (Rot). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

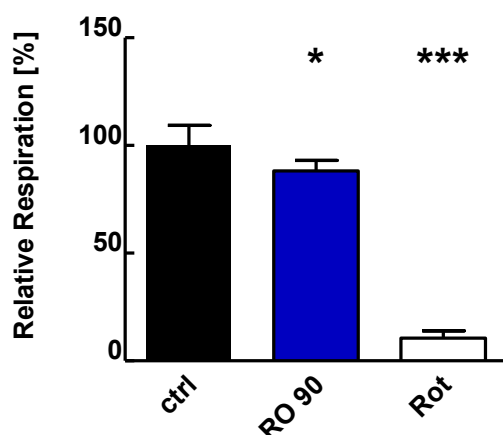


Figure 24: Respiration of isolated rat mitochondria is inhibited by RO 90-7501

Oxygen consumption (% control) of isolated rat liver mitochondria (n = 6) treated with 10 μ M RO 90-7501 (RO 90), 0.1 % DMSO (ctrl) or 1 μ M rotenone (Rot). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

The oxygen consumption of isolated rat liver mitochondria was significantly (p < 0.01) inhibited by 34.4 % after exposure to punicalagin (Figure 23). The oxygen consumption of isolated rat liver mitochondria was also significantly (p < 0.05) reduced by 11.8 % after exposure to 10 μ M RO 90-7501 (Figure 24). The inhibition occurred at complex I of the ETC within the mitochondria, which is indicated by the NAD-dependent substrates glutamate and malate.

3.8 Influence of punicalagin and RO 90-7501 on ATP levels of *C. elegans*

These findings suggest that with a temporary decrease in respiration after 6 h of treatment and an inhibition of complex I, the electron transfer over the ETC may be interrupted leading to a possible inhibition in the generation of ATP. ATP is formed during oxidative phosphorylation at the ETC within the mitochondria under consumption of oxygen. Therefore, the ATP content of *C. elegans* wild type nematodes was determined after treatment with 1 μ M punicalagin or 1 μ M Ro 90-7501 for different time periods.

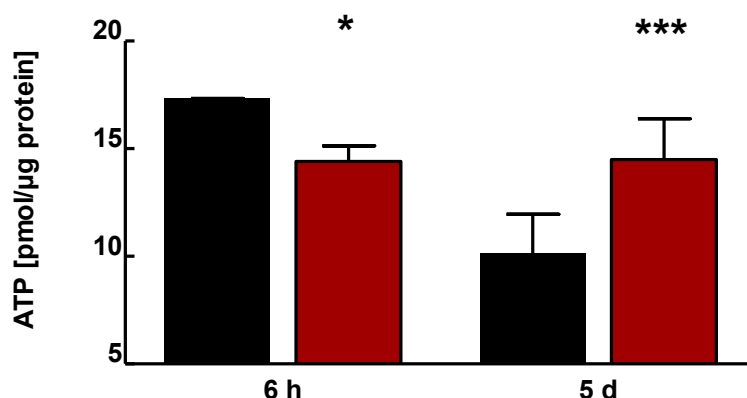


Figure 25: Modulation of ATP levels in *C. elegans* by punicalagin

ATP content (pmol/ μg) of nematodes (n = 3) after a 6 h and 5 d treatment with 1 μM punicalagin (red) or 0.1 % DMSO (black). Values are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After *C. elegans* wild type nematodes were treated with 1 μM punicalagin for 6 h; the ATP content was significantly (p < 0.05) decreased by 16.81 % (Figure 25). This short-term decrease may be caused by the inhibition of complex I of the ETC. After 5 d of treatment, the ATP content is significantly (p < 0.001) increased by 42.96 % in comparison to the control nematodes.

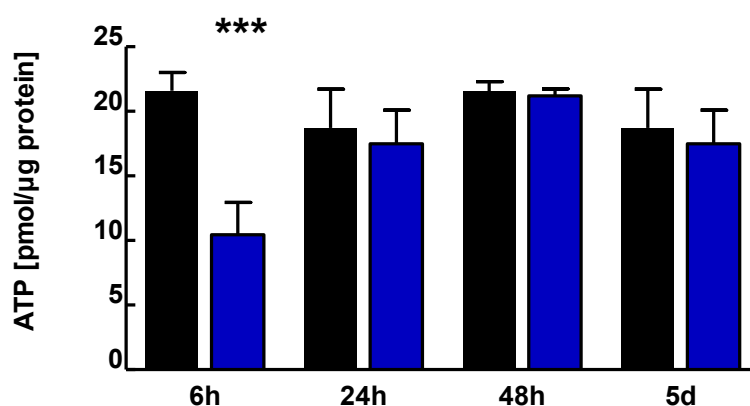


Figure 26: Modulation of ATP levels in *C. elegans* by RO 90-7501

ATP content (pmol/ μg) of nematodes (n = 3) after a 6 h, 24 h, 48 h and 5 d treatment with 1 μM RO 90-7501 (blue) or 0.1 % DMSO (black). Values are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After 6 h of exposure to 1 μM RO 90-7501, the ATP content of *C. elegans* wild type nematodes was significantly (p < 0.001) decreased by 48.40 % (Figure 26), which is possibly caused by the inhibition of complex I of the ETC. No

significant differences in ATP could be determined after 24 h, 48 h and 5 d of exposure in comparison to the untreated control nematodes.

3.9 Punicalagin decreases the glucose oxidation in *C. elegans*

To investigate whether the short-term decrease in ATP content is based on the inhibition of complex I and/or the resulting decrease in respiration, the glucose oxidation as a measure for glucose metabolism of *C. elegans* was determined by using labeled [^{14}C] D-glucose. The wild type nematodes were exposed to 1 μM punicalagin for 6 h before performing the assay.

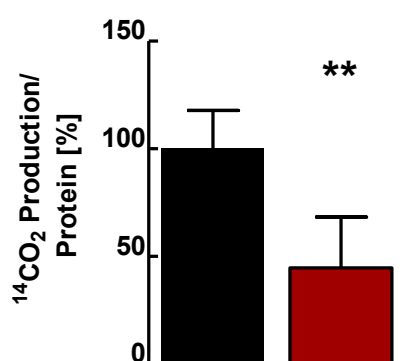


Figure 27: Punicalagin decreases glucose oxidation in *C. elegans*

$^{14}\text{CO}_2$ production (% control) of nematodes ($n = 6$) after a 6h treatment with 1 μM punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus respective controls.

The glucose oxidation in *C. elegans* was significantly ($p < 0.01$) decreased by 44.70 % (Figure 27) after treatment with 1 μM punicalagin as shown by impaired production of ^{14}C -labeled carbon dioxide.

3.10 Fat content and pumping rate of *C. elegans* are not influenced by punicalagin

Two other important metabolic parameters in *C. elegans* are the fat content and the pumping rate of the nematodes. The fat content reflects the metabolic state of *C. elegans*, the existence of drifts in the metabolic pathways, or maybe the presence of CR conditions. Wild type nematodes were exposed to 1 μ M punicalagin for 5 d and afterwards harvested for the determination of the fat content.

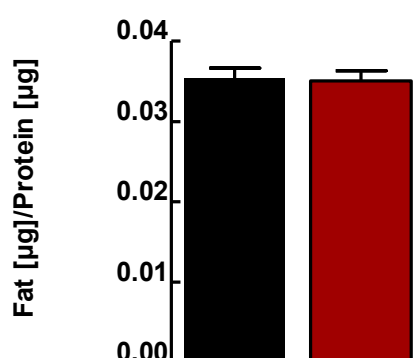


Figure 28: Punicalagin has no influence on fat content of *C. elegans*

Fat content of nematodes (n = 3) after 5 d of treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After 5 d of exposure to 1 μ M punicalagin there were no significant differences between the treatment and the control group (Figure 28). The fat content of *C. elegans* did not change under the influence of punicalagin.

The pumping rate reflects alterations in the food uptake. A reduction in food uptake induced by this compound may be the reason for the modulating effects of punicalagin. The determination of the pumping rate also indicates whether the compound itself is taken up by the nematodes or if, because of a different taste or smell, the nematodes refuse the uptake of punicalagin-containing food. Wild type nematodes were exposed to 1 μ M punicalagin for 10 d, and pharyngeal pumping was scored per minute.

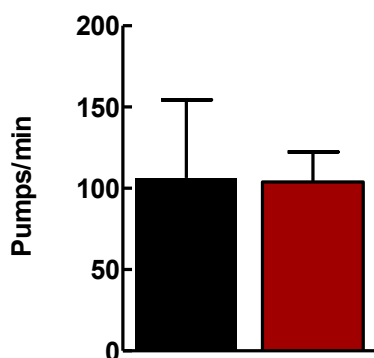


Figure 29: Pumping rate of *C. elegans* is not influenced by punicalagin

Pumping rate (pumps/ min) of nematodes (n = 10) after 10 d of treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

The pumping rate of the nematodes did not significantly change after 10 d of treatment with 1 μ M punicalagin (Figure 29) which is reflected by a similar rate of pharyngeal movements per minute compared to untreated nematodes.

3.11 AMPK/AAK-2 is involved in lifespan extension of *C. elegans* under the influence of punicalagin and RO 90-7501

AAK-2 (homologous to mammalian AMPK) is a sensor that couples lifespan to information about energy levels and insulin-like signals. The AMP:ATP ratio, a marker for energy levels, can be used to prognosticate life expectancy in *C. elegans* (Apfeld et al. 2004). Lifespan analyses in *aak-2* knock-out mutants of *C. elegans* were performed to discover the role of AAK-2 in lifespan extension afforded by punicalagin or RO 90-7501. Therefore, 1 μ M punicalagin or 1 μ M RO 90-7501 were applied to *aak-2* KO mutants from L4 larval stage to death and survival rate was analysed.

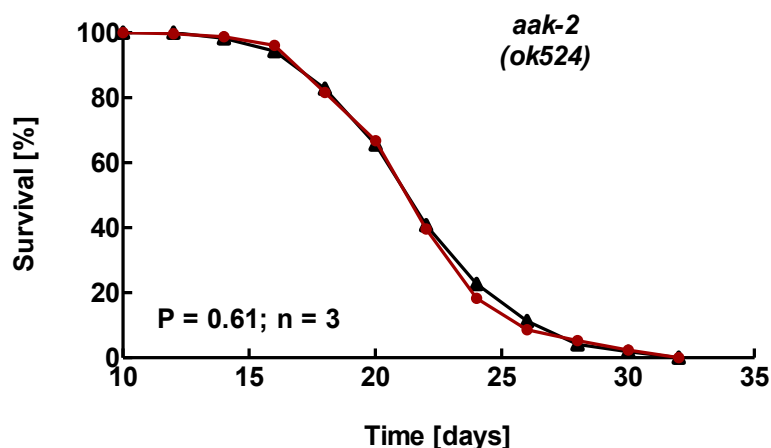


Figure 30: Lifespan extension mediated by Punicalagin is AAK-2-dependent

Lifespan analyses of *aak-2* KO nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

The lifespan-extending effect of 1 μ M punicalagin (Figure 30) and 1 μ M RO 90-7501 (Figure 31) is abolished in *aak-2* KO mutants. No significant differences were detected in comparison to untreated mutant nematodes. It can be assumed that the increased life expectancy mediated by both punicalagin and RO 90-7501 is AMPK/AAK-2-dependent.

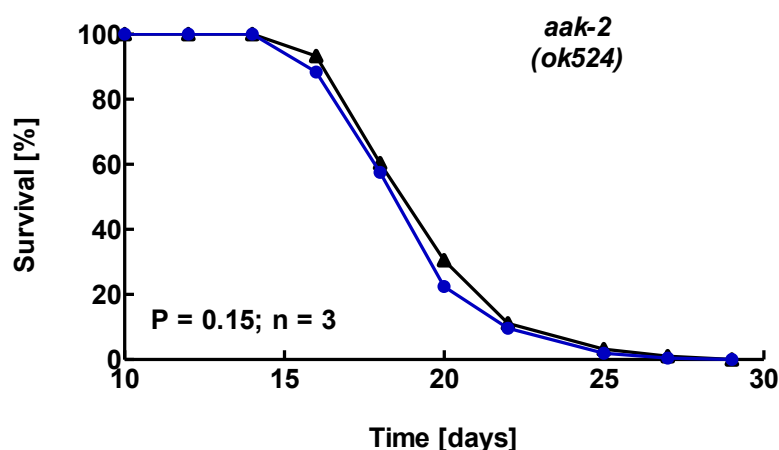


Figure 31: Lifespan extension mediated by RO 90-7501 is AAK-2-dependent

Lifespan analyses of *aak-2* KO nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

3.12 Modulation of ROS formation in *C. elegans* by punicalagin and RO 90-7501

Several agents produce complex I-dependent mitochondrial stress. For example, rotenone disrupts the electron transfer through the ETC by inhibiting complex I and therefore enhances the formation of ROS (Sharma et al. 2011). In this work, the formation of mitochondrial-derived ROS (mtROS) in *C. elegans* was determined *in vivo* by using Mitotracker Red dye. This reduced probe does not fluoresce until it enters living cells, where it is oxidised to a fluorescent mitochondrion-selective probe and remains in the mitochondria (Li and Xing 2011). In addition, the formation of hydrogen peroxide was measured by using Amplex Red dye. Amplex Red reacts with hydrogen peroxide to produce highly fluorescent resorufin, which is catalysed by the horseradish peroxidase (Dikalov et al. 2007). Wild type nematodes were exposed to 1 μ M punicalagin or 1 μ M RO 90-7501 for different time periods before the measurement.

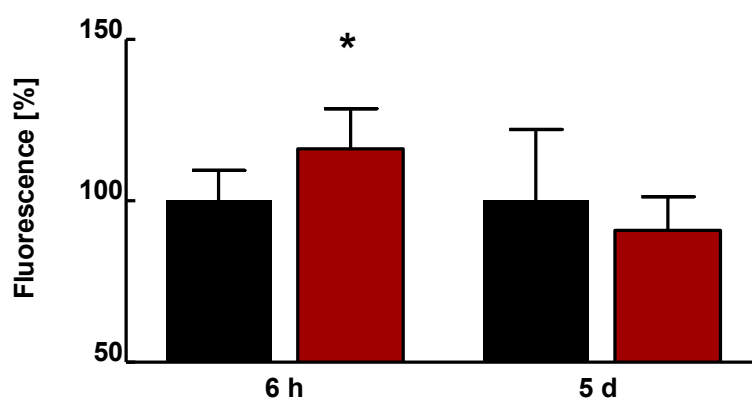


Figure 32: Modulation of mtROS formation in *C. elegans* after treatment with punicalagin MtROS formation (% control) of nematodes (n = 8) after 6 h and 5 d of treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After 6 h of treatment with 1 μ M punicalagin, a significant (p < 0.05) induction in the generation of mitochondrial-derived ROS by 16.10 % indicated *in vivo* by Mitotracker Red (Figure 32) was detected, which is shown as the fluorescence per μ g protein [%]. No significant differences in the formation of mtROS could be determined after 5 d of treatment compared to untreated nematodes.

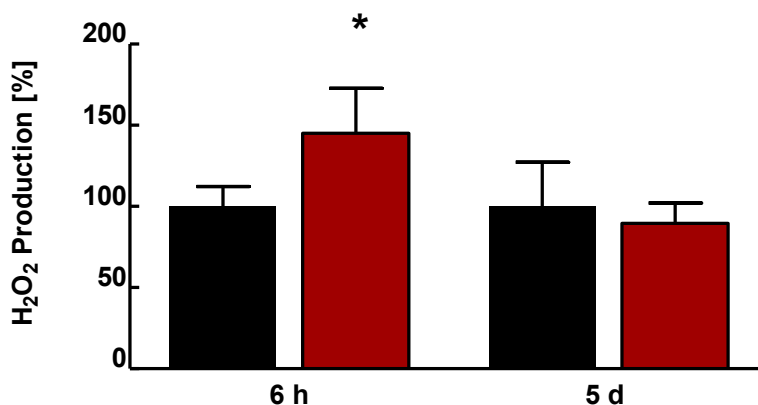


Figure 33: Modulation of H₂O₂ formation in *C. elegans* after treatment with punicalagin

Production of H₂O₂ (% control) of nematodes (n = 5) after 6 h and 5 d of treatment with 1 μM punicalagin (red) or 0.1 % DMSO (black). Values are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

The production of hydrogen peroxide in *C. elegans* wild type nematodes is also significantly (p < 0.05) increased by 45.16 % after a 6 h incubation with 1 μM punicalagin (Figure 33). No differences could be detected after 5 d in comparison to untreated nematodes.

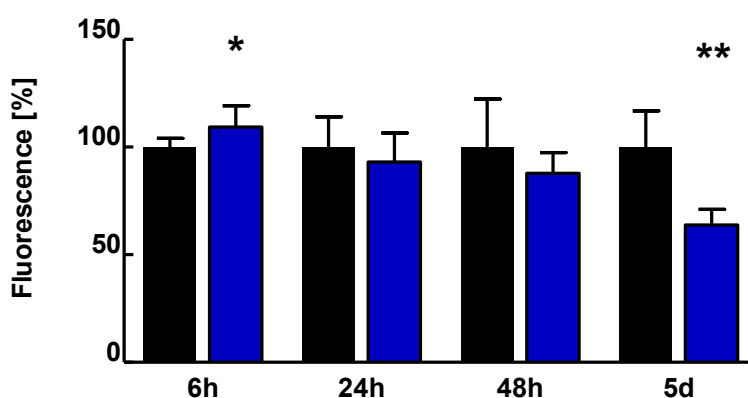


Figure 34: Modulation of mtROS formation in *C. elegans* after treatment with RO 90-7501

MtROS formation (% control) of nematodes (n = 8) after 6 h, 24 h, 48 h and 5 d of treatment with 1 μM RO 90-7501 (blue) or 0.1 % DMSO (black). Values are given as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

After 6 h of exposure to 1 μM RO 90-7501, a significant (p < 0.05) induction in the generation of mitochondrial-derived ROS of *C. elegans* by 9.25 % was detected, indicated *in vivo* by Mitotracker Red (Figure 34) and shown as fluorescence per μg protein [%]. No significant differences in the formation of

mtROS could be determined after 24 h and 48 h. After 5 d of treatment, a significant ($p < 0.01$) decrease in the formation of mtROS by 36.14 % was detected compared to untreated nematodes.

3.13 The lifespan-extending effect of punicalagin and RO 90-7501 in *C. elegans* is abolished by supplementation of an antioxidant

There are findings providing that antioxidant supplements which are capable to scavenge ROS, negatively influence the lifespan-extending effects of exercise or CR (Schulz et al. 2007). To determine whether ROS play a major role in lifespan extension mediated by punicalagin or RO 90-7501, lifespan analyses with additional supplementation of an antioxidant were performed. Therefore, wild type nematodes were treated with butylated hydroxyanisole (BHA) for 4 generations before starting the lifespan analyses. One μM punicalagin or RO 90-7501 were applied to the synchronised nematodes starting at L4 with or without additional BHA (10 μM).

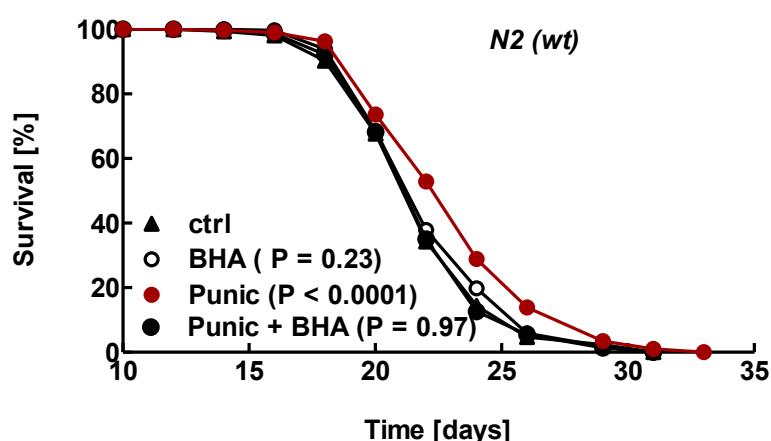


Figure 35: BHA abolishes the effect of punicalagin on lifespan of *C. elegans*

Lifespan analyses of N2 wild type nematodes exposed to DMSO, 10 μM BHA, 1 μM punicalagin (red) or 10 μM BHA + 1 μM Punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

The antioxidant BHA (10 μM) alone had no significant effect on lifespan of *C. elegans* wild type nematodes (Figure 35). Punicalagin resulted in a significant ($p < 0.0001$) increase in lifespan as observed before (Figure 12). The mean lifespan was extended by 1.24 d (5.56 %) and the maximum lifespan by 2 d (8.33 %). The combination of the antioxidant BHA (10 μM) and punicalagin

(1 μ M) did not significantly affect life expectancy. The effect of punicalagin was abolished by the additional supplementation of the antioxidant.

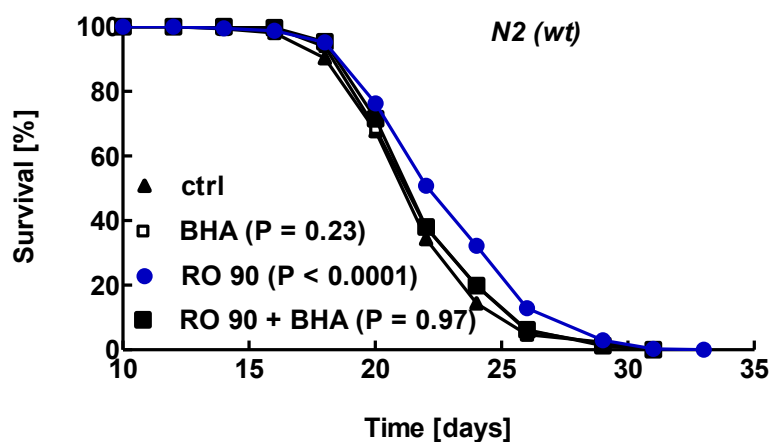


Figure 36: BHA abolishes the effect of RO 90-7501 on lifespan of *C. elegans*

Lifespan analyses of N2 wild type nematodes exposed to DMSO, 10 μ M BHA, 1 μ M RO 90-7501 (blue) or 10 μ M BHA + 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

The antioxidant BHA (10 μ M) alone had no significant effect on lifespan of *C. elegans* wild type nematodes (Figure 36). RO 90-7501 showed the significant ($p < 0.0001$) increase in lifespan as observed before (Figure 13). Mean lifespan was extended by 1.18 d (5.29 %), maximum lifespan by 2 d (8.33 %). The combination of BHA (10 μ M) and RO 90-7501 (1 μ M) did not affect life expectancy significantly. The effect of RO 90-7501 was abolished by the antioxidant. It can be assumed that the effect of both punicalagin and RO 90-7501 on lifespan of *C. elegans* is mediated by the transient increase of mtROS.

3.14 P38 MAPK/PMK-1 is involved in lifespan extension of *C. elegans*

ROS are sensed by p38 (mitogen-activated protein kinase) MAPK/PMK-1 (Inoue et al. 2005). In this work, the transient increase in mtROS induced by punicalagin and RO 90-7501 was found to play a major role in the extension of lifespan of *C. elegans*. A knock-out mutant for *pmk-1* was analysed for its role in mediating longevity of *C. elegans*. *Pmk-1* KO mutants were exposed to 1 μ M

punicalagin or 1 μ M RO 90-7501 from L4 to death, and survival rate was recorded.

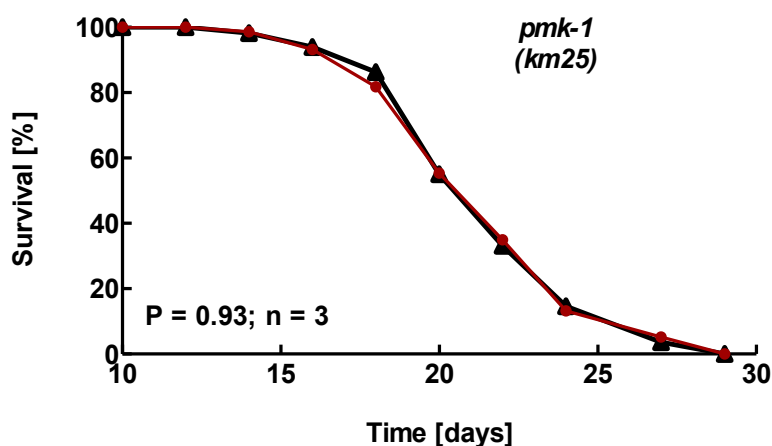


Figure 37: Punicalagin-induced longevity of *C. elegans* requires PMK-1

Lifespan analyses of *pmk-1* KO nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

The lifespan-extending effect of 1 μ M punicalagin is abolished in the *pmk-1* KO mutant of *C. elegans* (Figure 37). PMK-1 is involved in signaling pathways leading to longevity of *C. elegans* under the influence of punicalagin.

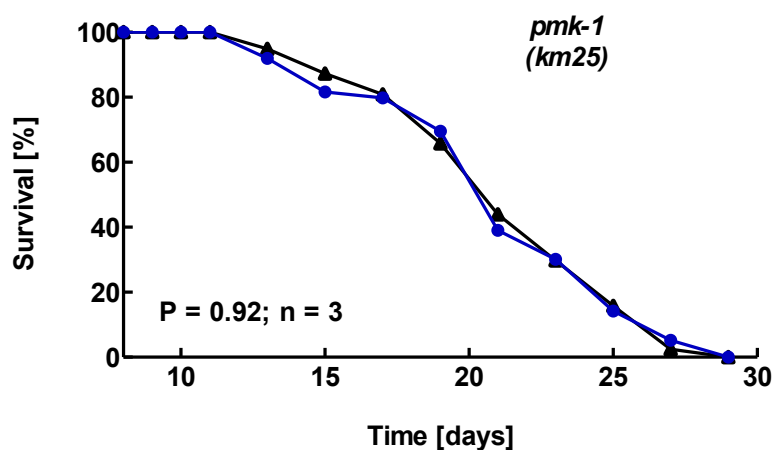


Figure 38: RO 90-7501-induced longevity of *C. elegans* requires PMK-1

Lifespan analyses of *pmk-1* KO nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

The effect on lifespan of *C. elegans* mediated by RO 90-7501 is abolished in *pmk-1* KO mutants (Figure 38) as reflected by a similar survival rate compared

to untreated nematode mutants. The stress kinase PMK-1 is involved in lifespan extension induced by RO 90-7501.

3.15 The lifespan extension of *C. elegans* is mediated by Nrf2/SKN-1

In *C. elegans*, SKN-1 (homologous to mammalian Nrf2) functions to resist oxidative stress, it regulates detoxification enzymes and initiates the adaptive response (Park et al. 2009). SKN-1 is present in nuclei of ASI neurons under normal conditions as well as in the intestine nuclei under conditions of stress. PMK-1 can directly phosphorylate SKN-1 under conditions of oxidative stress leading to translocation to nuclei of intestinal cells (Hayes 2009). A global *skn-1* KO mutant was tested in lifespan analyses for the involvement of SKN-1 in lifespan extension afforded by punicalagin and RO 90-7501.

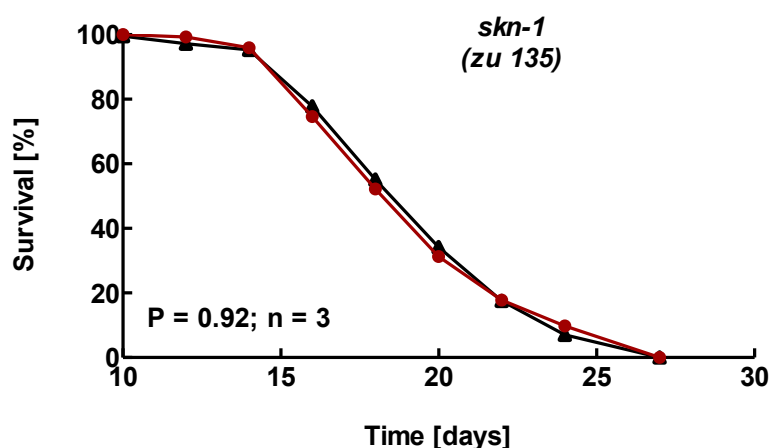


Figure 39: Lifespan extension of *C. elegans* by punicalagin is mediated by *skn-1* (zu135)

Lifespan analyses of *skn-1* (zu135, global KO) nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

In the *skn-1* global KO mutant of *C. elegans*, the lifespan-extending effect of punicalagin is abolished (Figure 39) as reflected by a similar survival rate compared to untreated mutant nematodes. The transcription factor SKN-1 is generally involved in the lifespan extension mediated by punicalagin.

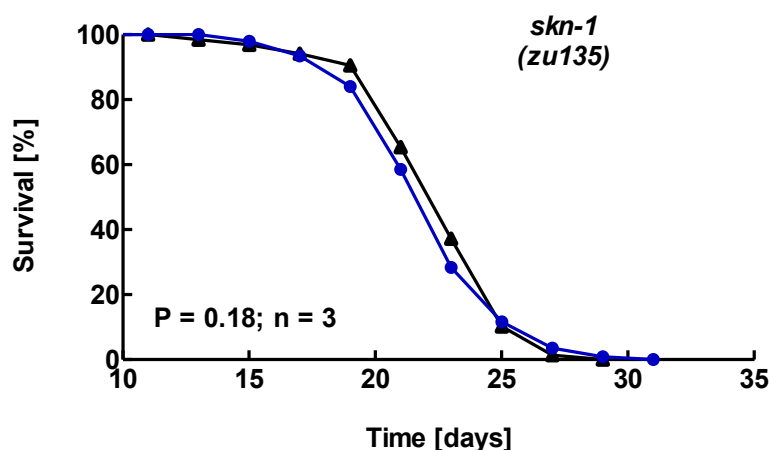


Figure 40: Lifespan extension of *C. elegans* by RO 90-7501 is mediated by *skn-1* (zu135)

Lifespan analyses of *skn-1* (zu135, global KO) nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

The transcription factor SKN-1 is also involved in the lifespan extension following exposure to 1 μ M RO 90-7501 (Figure 40). The lifespan-extending effect of RO 90-7501 is abolished in the *skn-1* global KO mutant of *C. elegans*.

In adult nematodes, SKN-1 is expressed in the ASI neurons as well as in the intestine (Bishop and Guarente 2007). The findings on global knock-out of *skn-1* suggest that SKN-1 is involved in the modulation of lifespan. With the help of another mutant, the *skn-1* (zu67), a knock-out for only SKN-1 expressed in the intestine, it could be possible to figure out which localization of SKN-1 is most responsible for the lifespan-extending effect induced by punicalagin or RO 90-7501. Lifespan analyses with *skn-1* (zu67) KO mutants were performed and survival rates were determined.

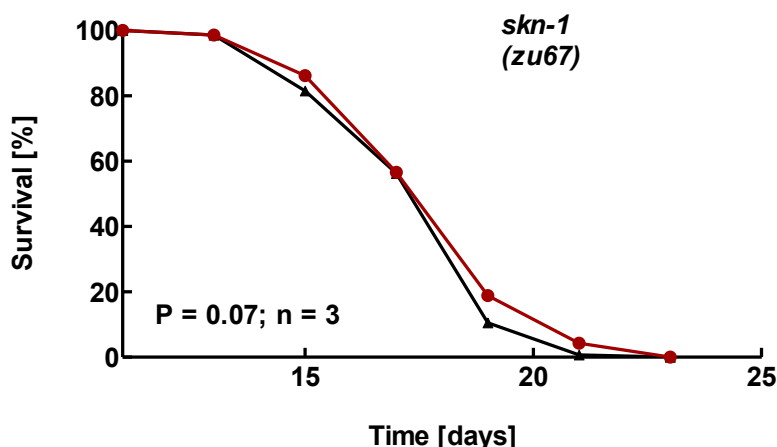


Figure 41: Lifespan extension of *C. elegans* by punicalagin is mediated by *skn-1* (zu67)

Lifespan analyses of *skn-1* (zu67, KO in the intestine) nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

The lifespan-extending effect induced by punicalagin is abolished in *skn-1* (zu67) KO mutants (Figure 41). The transcription factor SKN-1 expressed in the intestine is involved in the lifespan extension following treatment with 1 μ M punicalagin.

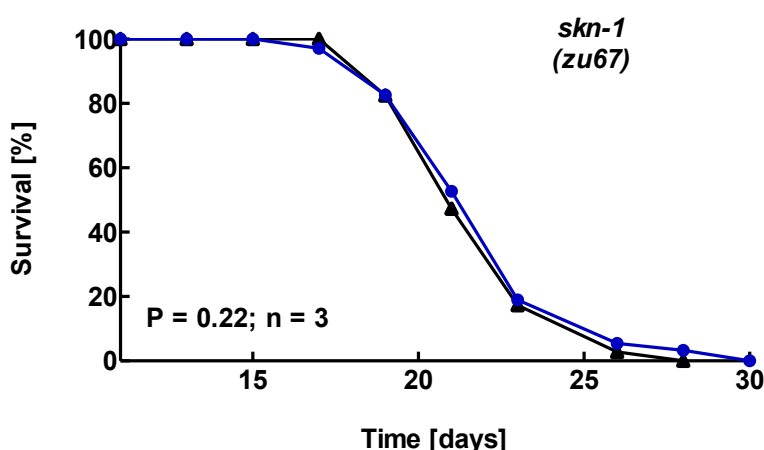


Figure 42: Lifespan extension of *C. elegans* by RO 90-7501 is mediated by *skn-1* (zu67)

Lifespan analyses of *skn-1* (zu67, KO in the intestine) nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

The enhancing effect on lifespan following exposure to RO 90-7501 is also mediated by SKN-1 expressed in the intestine. The lifespan extension is

abolished in a *skn-1* (zu67) KO mutant of *C. elegans* (Figure 42) as reflected by similar survival rates compared to untreated mutant nematodes.

Because no mutant with a knock-out in the ASI neurons is available, a global *skn-1* KO strain using an integrated transgene to restore the SKN-1 function in the intestine was used to test if SKN-1 expressed in the neurons is also mediating the effect of both compounds (Powolny et al. 2011). Therefore, another lifespan analyses was performed. *Skn-1* (zu135, global KO, restored in the intestine) mutants were exposed to 1 μ M punicalagin or 1 μ M RO 90-7501 from L4 to death, and survival rates were scored.

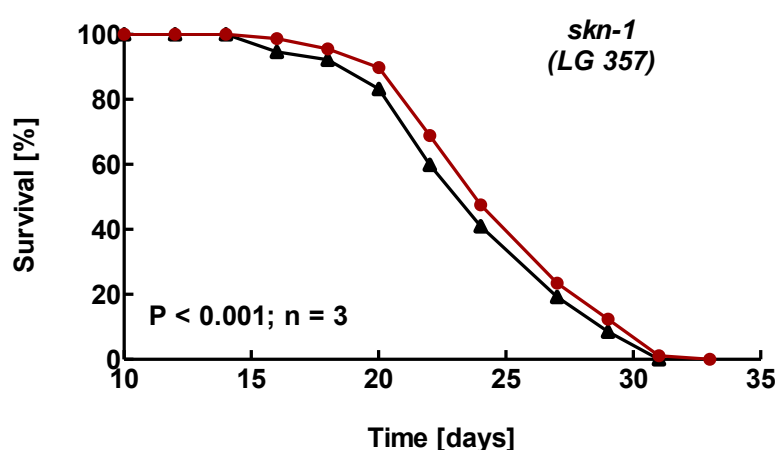


Figure 43: Lifespan extension of *C. elegans* by punicalagin is not mediated by *skn-1* (zu135 /*nT1[qIs51]*; *gels10[ges-1p::skn-1c::gfp]*; *rol-6(su1006)*)

Lifespan analyses of *skn-1* (zu135, global KO, restored in the intestine) nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

In *skn-1* (zu135 /*nT1[qIs51]*; *gels10[ges-1p::skn-1c::gfp]*; *rol-6(su1006)*), a global knock-out mutant for *skn-1* with a restored expression in the intestine, the lifespan-extending effect of punicalagin still occurred (Figure 43). There was a significant ($p < 0.001$) increase in lifespan reflected by an increased survival rate of the mutant nematodes treated with 1 μ M punicalagin. The mean lifespan was extended by 0.94 d (3.83 %), the maximum lifespan by 1.3 d (4.82 %). The lifespan extension caused by punicalagin is not mediated by SKN-1 expressed in the neurons.

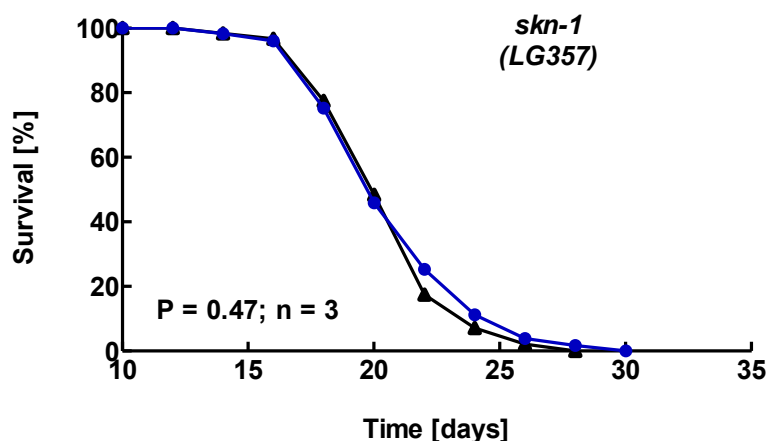


Figure 44: Lifespan extension of *C. elegans* by RO 90-7501 is not mediated by *skn-1* (*zu135 /nT1[qIs51];gels10[ges-1p::skn-1c::gfp; rol-6(su1006)*)

Lifespan analyses of *skn-1* (*zu135*, global KO, restored in the intestine) nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

In *skn-1* (*zu135 /nT1[qIs51];gels10[ges-1p::skn-1c::gfp; rol-6(su1006)*), a global knock-out mutant for *skn-1* with a restored expression in the intestine, the lifespan-extending effect of RO 90-7501 was abolished (Figure 44). SKN-1 expressed in the neurons is also involved in the lifespan extension induced by RO 90-7501.

3.16 The role of FOXO/DAF-16 for lifespan extension of *C. elegans*

FOXO/DAF-16 is another important transcription factor which can influence longevity. DAF-16 functions in *C. elegans* insulin/IGF1 signaling pathway and promotes longevity (Greer and Brunet 2009). Therefore, the role of DAF-16 in lifespan extension of *C. elegans* mediated by punicalagin and RO 90-7501 was determined by performing lifespan analyses with a *daf-16* KO mutant. Nematodes were exposed to 1 μ M punicalagin or 1 μ M RO 90-7501 and survival rates were analysed.

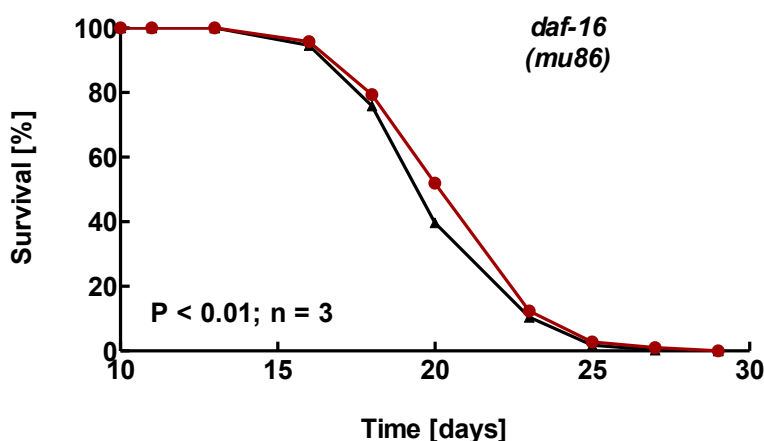


Figure 45: Lifespan extension mediated by punicalagin is not DAF-16-dependent

Lifespan analyses of *daf-16* KO nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

The lifespan extension induced by punicalagin is not mediated by DAF-16. One μ M punicalagin is still able to extend lifespan significantly ($p < 0.01$) in *daf-16* KO mutants (Figure 45). The mean lifespan is extended by 0.55 d (2.63 %). The transcription factor DAF-16 is not involved in the signal pathway induced by punicalagin.

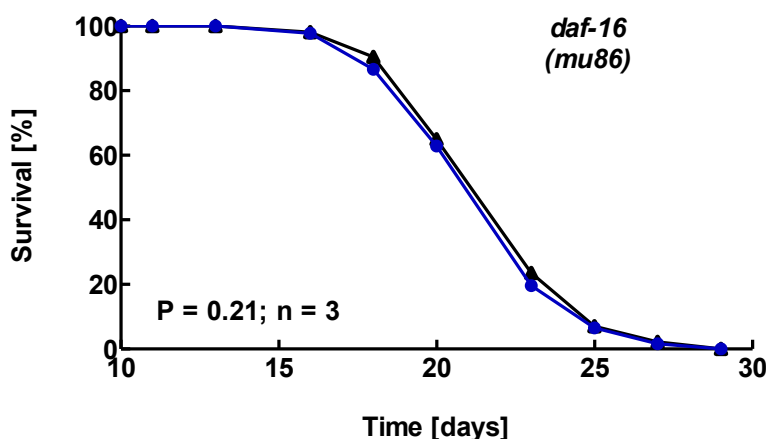


Figure 46: Lifespan extension mediated by RO 90-7501 is DAF-16-dependent

Lifespan analyses of *daf-16* KO nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

Contrary to punicalagin the lifespan-extending effect of RO 90-7501 is abolished in *daf-16* KO mutants (Figure 46), which is reflected by a similar survival rate in comparison to untreated mutant nematodes. The transcription factor DAF-16 is involved in lifespan extension promoted by RO 90-7501.

3.17 The modulation of antioxidant enzyme activities in *C. elegans* by punicalagin and RO 90-7501

The superoxide dismutase (SOD) is an enzyme detoxifying superoxide radicals (Van Raamsdonk and Hekimi 2012), one group of ROS. Another enzyme being part of the detoxifying system is catalase (CTL), which detoxifies the generated hydrogen peroxide (Gems and Doonan 2009). Both antioxidant enzymes were tested for their changes in activity under the influence of punicalagin or RO 90-7501. Therefore, *C. elegans* wild type nematodes were exposed to 1 μ M punicalagin for 5 d or 1 μ M RO 90-7501 for 24 h and the activity of SOD and CTL was measured by using standard assay kits.

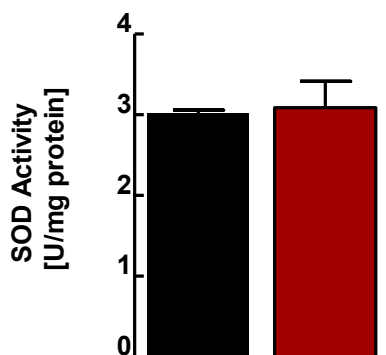


Figure 47: Punicalagin does not influence SOD activity of *C. elegans*

SOD activity (U/mg protein) of nematodes (n = 3) after 5 days of treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

5 d of exposure to 1 μ M punicalagin did not significantly influence the activity of SOD (Figure 47).

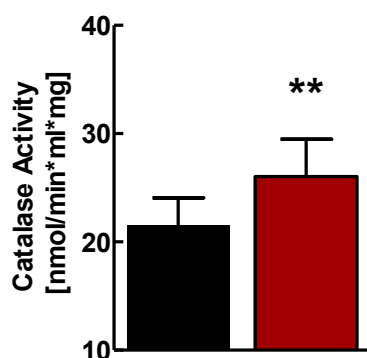


Figure 48: Treatment with punicalagin induces the catalase activity of *C. elegans*

Catalase activity (nmol/ min*ml*mg protein) of nematodes (n = 3) after 5 days of treatment with 1 μ M punicalagin (red) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

The activity of catalase was significantly (p < 0.01) induced by 21.15 % after exposure to 1 μ M punicalagin for 5 d (Figure 48).

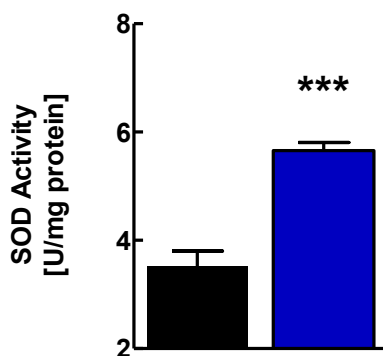


Figure 49: RO 90-7501 induces the SOD activity of *C. elegans*

SOD activity (U/mg protein) of nematodes (n = 3) after 24 h of treatment with 1 μ M RO 90-7501 (blue) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

SOD activity was significantly (p < 0.001) increased by 60.85 % after 24 h of exposure to 1 μ M RO 90-7501 (Figure 49).

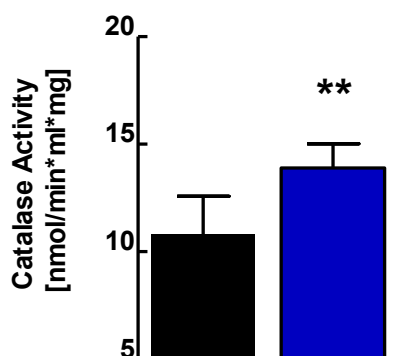


Figure 50: Treatment with RO 90-7501 induces the catalase activity of *C. elegans*

Catalase activity (nmol/min*ml*mg protein) of nematodes (n = 3) after 24 h of treatment with 1 μ M RO 90-7501 (blue) or 0.1 % DMSO (black). Values are given as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus respective controls.

Treatment with 1 μ M RO 90-7501 also significantly (p < 0.01) induced the activity of catalase by 28.72 % after 24 h of exposure (Figure 50).

3.18 Punicalagin and RO 90-7501 modulate the protein homeostasis in *C. elegans*

Several compounds (including RO 90-7501) have protein-aggregat-binding capacities and are able to reduce age-dependent misfolding and accumulation of proteins (Bohrmann et al. 2000). A *C. elegans* model of human proteotoxic disease was used to determine the possible influence of punicalagin or RO 90-7501 on the formation and accumulation of protein aggregates. The transgenic strain CI 4176 [*smg-1(cc546)* I; *dvIs27* X] expresses muscle-specific A β ₁₋₄₂ with raising the temperature to 25 °C. The control strain CI 802 [*smg-1(cc546)* I; *rol-6(su1006)* II] also contains this transgene, but does not undergo paralysis with temperature up-shift (Dostal and Link 2010). Transgenic nematodes were exposed to 1 μ M punicalagin or 1 μ M RO 90-7501 and paralysis assays were performed as described earlier.

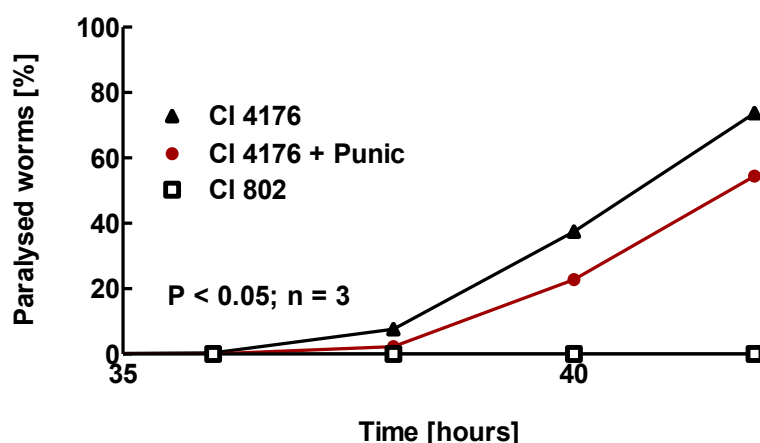


Figure 51: Punicalagin slows protein aggregation in a transgenic *C. elegans* model of human proteotoxic disease

Paralysed nematodes [%] after up-shift of temperature to 25 °C with punicalagin treatment (red, $n = 300$) vs. control (black, $n = 300$). Values are given as mean out of 3 independent experiments.

Transgenic nematodes CI 4176 [*smg-1(cc546)* I; *dvIs27* X] had a significant decreased ($p < 0.05$) paralysis compared to untreated transgenic nematodes after exposure to 1 μ M punicalagin (Figure 51).

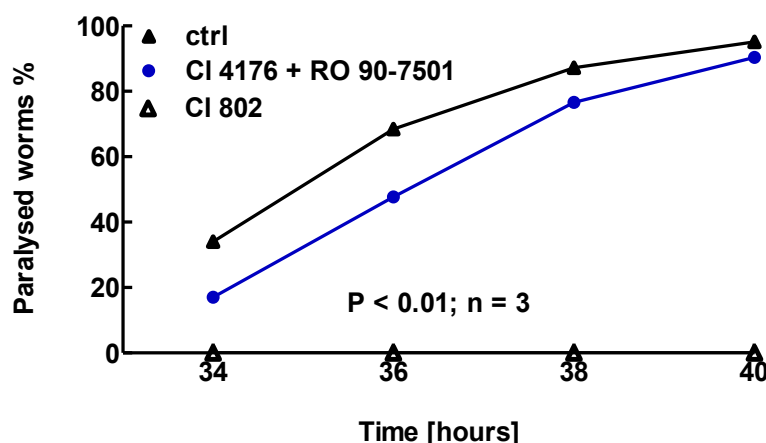


Figure 52: RO 90-7501 slows protein aggregation in a transgenic *C. elegans* model of human proteotoxic disease.

Paralysed nematodes [%] after upshift of temperature to 25 °C with RO 90-7501 treatment (blue, n = 300) vs. control (black, n = 300). Values are given as mean out of 3 independent experiments.

Transgenic nematodes CI 4176 [*smg-1(cc546)* I; *dvls27* X] showed a significant decrease ($p < 0.01$) in paralysis compared to untreated nematodes after exposure to 1 μ M RO 90-7501 (Figure 52). Protein aggregation occurring in *C. elegans* muscle cells was slowed under punicalagin and RO 90-7501 treatment. As expected, the transgenic control strain showed no paralysis after the temperature upshift.

The *C. elegans* transcription factor HSF-1 (homologous to mammalian HSF1) activates the transcription of heat shock genes in response to heat or oxidative stress, which encode chaperones and proteases to preserve proteostasis (Rodriguez et al. 2013). To analyse the role of the transcription factor HSF-1 in lifespan extension induced by punicalagin or RO 90-7501, lifespan analyses with *hsf-1* knock-out mutants were performed and survival rates were recorded.

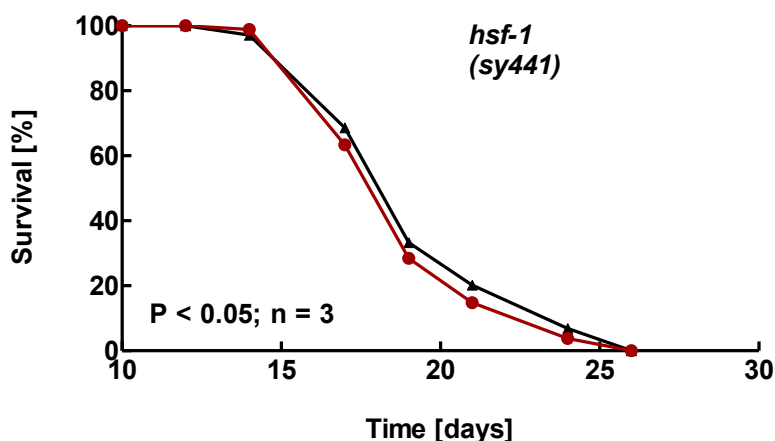


Figure 53: Lifespan extension of *C. elegans* induced by punicalagin is HSF-1-dependent

Lifespan analyses of *hsf-1* KO nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

Treatment of 1 μ M punicalagin significantly ($p < 0.05$) shortened the lifespan of *C. elegans* *hsf-1* KO mutants (Figure 53), which is reflected by a shift of the survival curve to the left. The mean lifespan was shortened by 0.48 d (2.48 %), the maximum lifespan by 0.7 d (3.45 %). The transcription factor HSF-1 plays an important role in punicalagin-induced longevity of *C. elegans*.

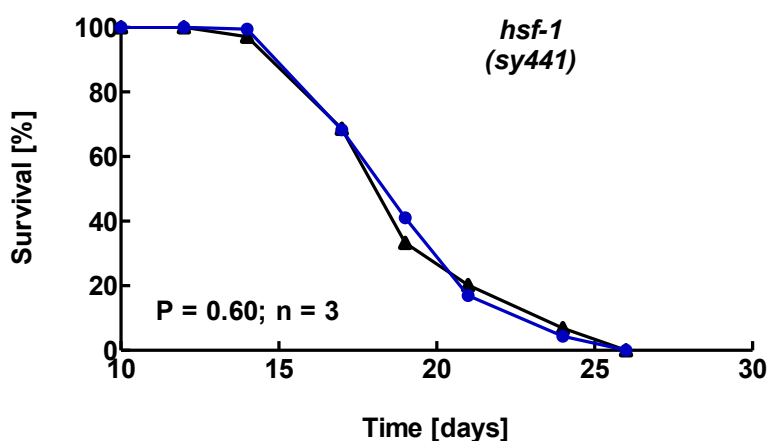


Figure 54: Lifespan extension mediated by RO 90-7501 is HSF-1-dependent

Lifespan analyses of *hsf-1* KO nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

The lifespan-extending effect induced by exposure to 1 μ M RO 90-7501 is abolished in *hsf-1* KO mutants of *C. elegans* (Figure 54). The transcription factor HSF-1 is involved in lifespan extension mediated by RO 90-7501.

AIP-1 (homologous to mammalian AIRAP) is one possible target of HSF1/HSF-1 and adapts the protein degradation machinery of the cell (Calabrese 2005). Lifespan analyses with *aip-1* knock-out mutants were performed. Therefore nematodes were exposed to 1 μ M punicalagin or 1 μ M RO 90-7501 and survival rates were analysed.

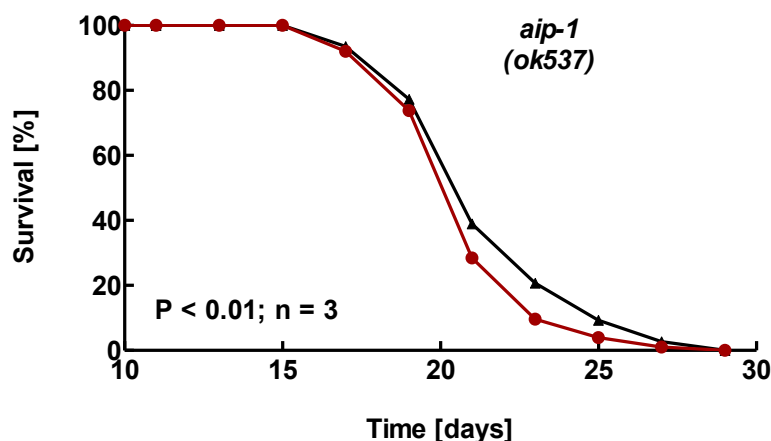


Figure 55: Lifespan extension of *C. elegans* mediated by punicalagin is AIP-1-dependent

Lifespan analyses of *aip-1* KO nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

A significant ($p < 0.01$) lifespan-shortening effect occurred in an *aip-1* KO mutant of *C. elegans* after exposure to 1 μ M punicalagin (Figure 55), represented by a shift of the survival curve to the left. The mean lifespan was shortened by 0.64 d (3.0 %), the maximum lifespan by 1.4 d (6.28 %). AIP-1 is required for the lifespan-extending effect in *C. elegans* induced by punicalagin.

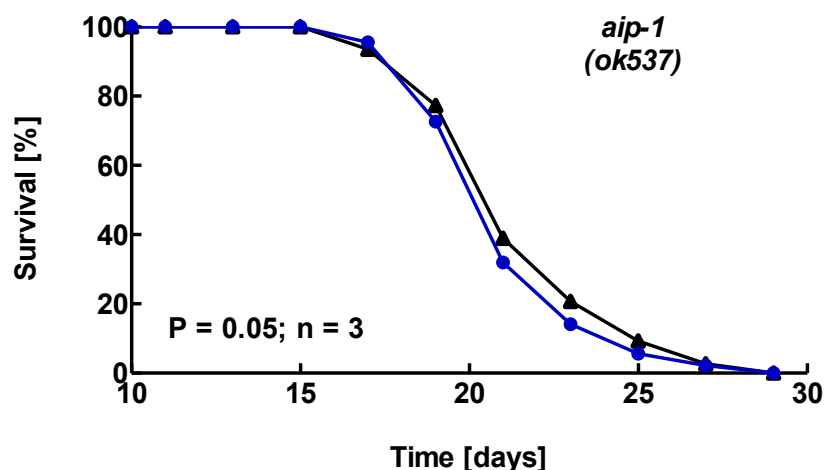


Figure 56: Lifespan extension mediated by RO 90-7501 is AIP-1-dependent

Lifespan analyses of *aip-1* KO nematodes in the presence (blue) or absence (black) of 1 μ M RO 90-7501. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 3.

The lifespan-extending effect of 1 μ M RO 90-7501 was abolished in *aip-1* KO mutants of *C. elegans* (Figure 56). No significant difference in the survival rate compared to untreated control mutants was observed. The extension of lifespan after exposure to RO 90-7501 is mediated by AIP-1.

3.19 The UPR_{ER} is involved in punicalagin- and RO 90-7501-mediated longevity of *C. elegans*

The unfolded protein response (UPR) is a signaling pathway which is induced by accumulation of misfolded proteins in the endoplasmic reticulum (ER). There are 3 transmembrane sensors existing in *C. elegans*, PERK/PEK-1, IRE1/IRE-1 and ATF6/ATF-6 (Richardson et al. 2011). Splicing of XBP1/XBP-1 mRNA is IRE-1-mediated and required for survival under ER stress. The IRE-1/XBP-1 pathway is acting complementary with PEK-1. Transcription of the chaperone HSP-4, one homolog of the mammalian BIP/GRP78, is induced upon ER stress. The activating transcription factor 4 ATF4/ATF-5 is a downstream target of PEK-1 and is translated more efficiently to initiate protective or apoptotic signaling (Shen et al. 2001). Knock-out mutants for *hsp-4*, *atf-6*, *pek-1*, *ire-1* and *xbp-1* were tested for their role in the pathway leading to longevity of *C. elegans* after exposure to punicalagin or RO 90-7501. Life span analyses were performed and survival rates were analysed.

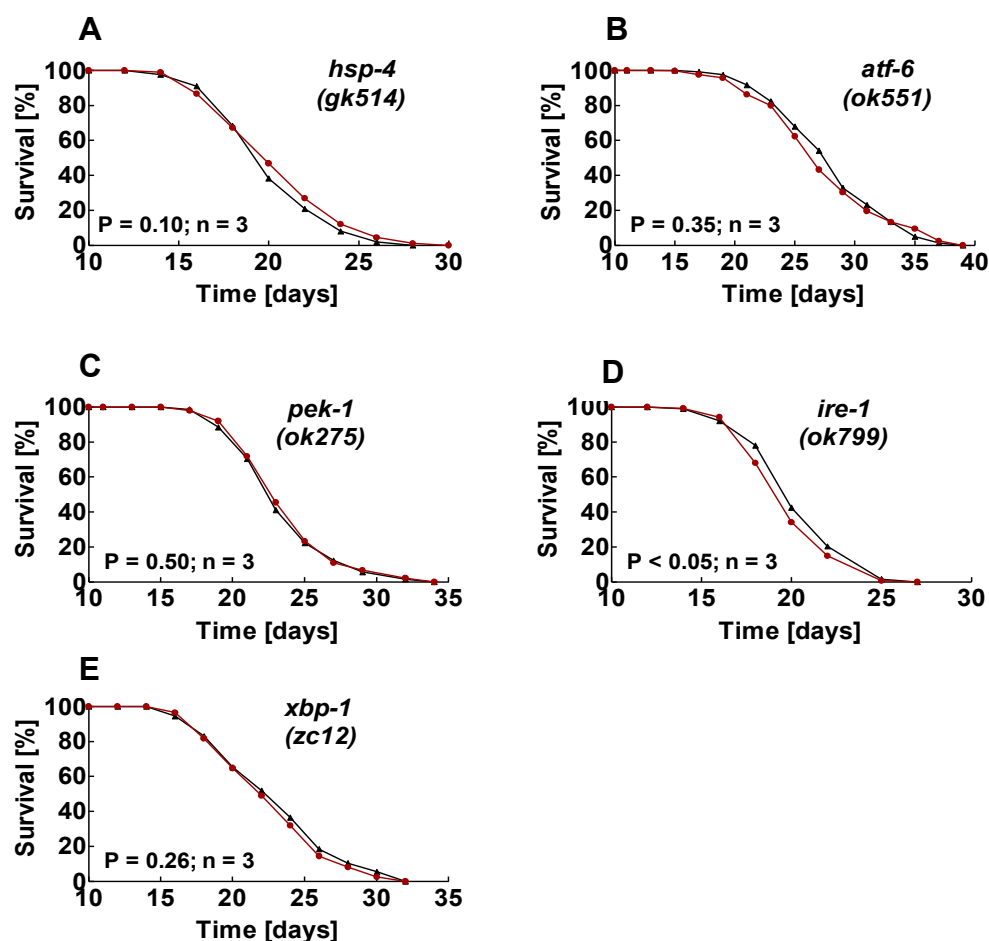


Figure 57: Lifespan modulation of UPR_{ER} KO mutants of *C. elegans* after punicalagin treatment

Lifespan analyses of UPR_{ER} KO mutant nematodes in the presence (red) or absence (black) of 1 μ M punicalagin. **A.** *hsp-4* (gk514) KO **B.** *atf-6* (ok551) KO **C.** *pek-1* (ok275) KO **D.** *ire-1* (ok799) KO **E.** *xbp-1* (zc12) KO. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan \pm SEM can be found in supplemental table 2.

No significant differences in survival rates of *hsp-4*, *atf-6*, *pek-1* and *xbp-1* KO mutants exposed to 1 μ M punicalagin compared to untreated control mutants were detected (Figure 57). HSP-4, ATF-6, PEK-1 and XBP-1 play a role in the punicalagin-mediated longevity of *C. elegans*. The lifespan of the knock-out of *ire-1*, the gene encoding for one of the transmembrane sensors of the ER, was significantly ($p < 0.05$) shortened under exposure to 1 μ M punicalagin, represented by a shift of the survival curve to the left. The mean lifespan was decreased by 0.52 d (2.55 %). IRE-1, as one part of the UPR_{ER} is also involved in the lifespan extension induced by punicalagin.

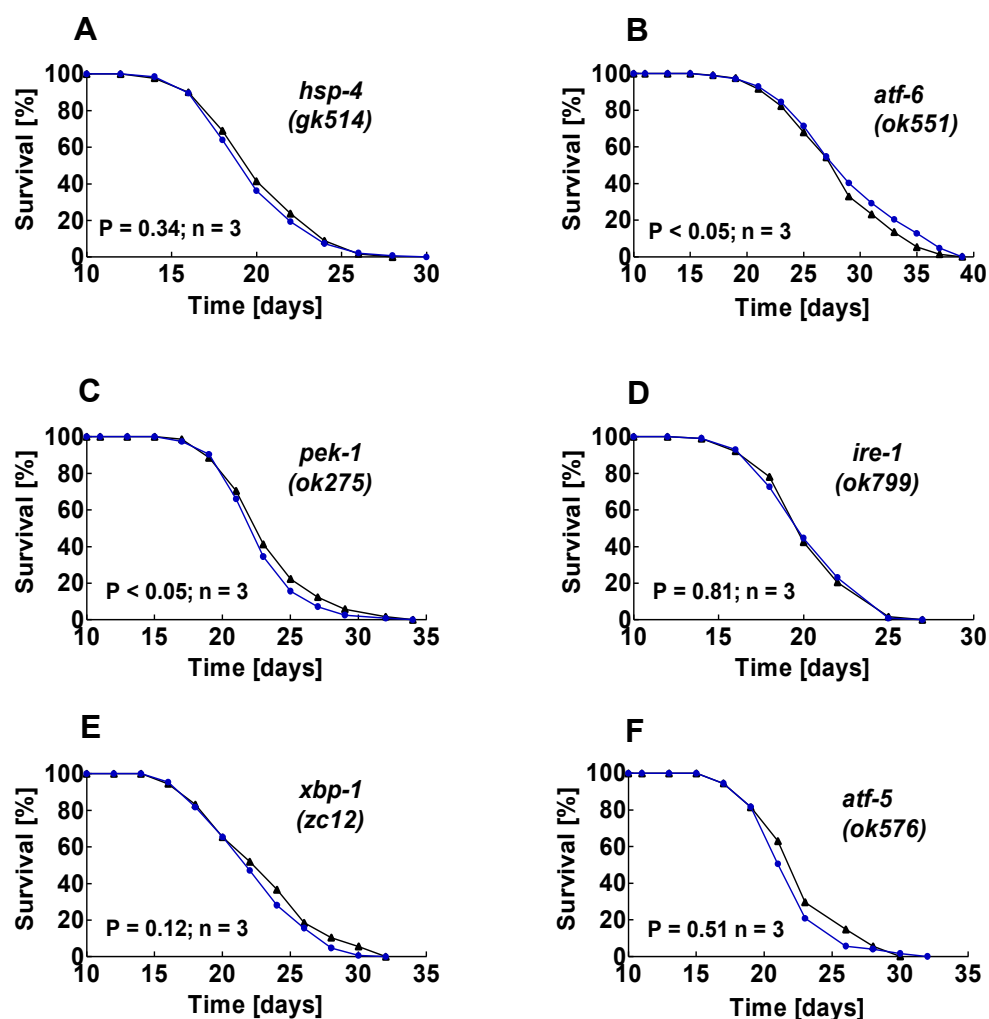


Figure 58: Lifespan modulation of UPR_{ER} KO mutants of *C. elegans* after RO 90-7501 treatment

Lifespan analyses of UPR_{ER} KO mutant nematodes in the presence (blue) or absence (black) of 1 μM RO 90-7501 **A.** *hsp-4* (*gk514*) KO **B.** *atf-6* (*ok551*) KO **C.** *pek-1* (*ok275*) KO **D.** *ire-1* (*ok799*) KO **E.** *xbp-1* (*zc12*) KO **F.** *atf-5* (*ok576*) KO. Values are given as mean out of 3 independent experiments. Details to mean and maximum lifespan ± SEM can be found in supplemental table 3.

No significant differences in survival rates of *hsp-4*, *ire-1*, *atf-5* and *xbp-1* KO mutants exposed to 1 μM RO 90-7501 compared to untreated control mutants were detected (Figure 58). HSP-4, IRE-1, ATF-5 and XBP-1 play a role in the RO 90-7501-mediated longevity of *C. elegans*. The lifespan of the knock-out of *pek-1* is significantly ($p < 0.05$) shorten under exposure to 1 μM RO 90-7501, represented with a shift of the survival curve to the left. The mean lifespan was decreased by 0.55 d (2.35 %), the maximum lifespan by 0.7 d (2.80 %).

PEK-1, as one transmembrane sensor of the UPR_{ER} is also involved in the lifespan extension induced by RO 90-7501. The *atf-6* knock-out mutant nematodes exposed to 1 μ M RO 90-7501 showed a significant ($p < 0.05$) increase in survival rate compared to untreated worms. The mean lifespan was extended by 0.76 d (2.68 %), the maximum lifespan by 2 d (6.45 %). The transmembrane sensor ATF-6 seems to play no role in the longevity of *C. elegans* mediated by RO 90-7501.

4. Discussion

The investigation of natural or synthetic compounds that can promote health and decelerate ageing are of ongoing scientific interest. In this work, two compounds, which were previously found to activate mitochondrial metabolism indicated by an increased ATP content in a cell-based screening, were investigated for their ability to extend lifespan in the roundworm *C. elegans*. Punicalagin is a natural ellagitannin from the pomegranate (Heber 2011), and RO 90-7501 is a pharmaceutical compound previously identified to diminish protein toxicity induced by A β ₄₂ *in vitro* (Bohrmann et al. 2000). Both were examined regarding their potentially modulating effects on the metabolism of *C. elegans*.

Polyphenols from plants have health beneficial and anti-ageing effects. In *C. elegans* they extend the lifespan as described for aspalathin from rooibos (Chen et al. 2012), apple polyphenols (Sunagawa et al. 2011), catechin (Saul et al. 2009), and blueberry polyphenols (Wilson et al. 2006). In this work, punicalagin also was found to significantly extend lifespan of *C. elegans*. The same applied for the second examined compound, RO 90-7501. The lifespan-modulating effects of different concentrations were tested, but 1 μ M was the most effective for both. Punicalagin spontaneously hydrolyses into smaller phenolic compounds such as ellagic acid (Larrosa et al. 2006). Ellagic acid is also known to have health-beneficial effects (Panchal et al. 2013). This raises the question, if the lifespan-extending effect of the punicalagin treatment in *C. elegans* is potentially ellagic acid-mediated. Although ellagic acid can increase the lifespan of *C. elegans* at higher concentrations (Saul et al. 2011), in this work, no lifespan-extending effect for ellagic acid could be determined in the concentration used for punicalagin, 1 μ M in the agar. Since punicalagin significantly does extend lifespan at that concentration, it can be assumed that punicalagin itself is responsible for the lifespan-enhancing effect.

Consequently, the question arose, whether the punicalagin- and RO 90-7501-mediated longevity also correlates with an improved quality of life. Locomotion, a parameter representing the mobility and behavior of the nematodes, was determined after long-term exposure to both compounds.

Punicalagin and RO 90-7501 significantly increased locomotion after 10 d (punicalagin) and 8 d (RO 90-7501) of exposure. Treated nematodes were able to cover a longer distance in a defined time frame in comparison to untreated nematodes, reflecting an improved fitness at an advanced age. The improved fitness can be regarded as the reason for or even the consequence of longevity. Slowing down ageing by interventions can improve fitness at an advanced age, or increased fitness itself leads to longevity by activating signal transduction pathways that stimulate the antioxidant system (Traustadottir et al. 2012).

Longevity is not only correlated with improved fitness, it is also linked with resistance against oxidative and/or thermal stress (Johnson et al. 1996; Lithgow et al. 1995). Lifespan extension can be the reason or the consequence of stress resistance. Nematodes treated with RO 90-7501 showed a significant increase in survival under oxidative stress generated by paraquat, and under thermal stress exhibited by the up-shift of temperature to 37°C. The toxicity of paraquat is caused by massive formation of superoxide radicals (Bagley et al. 1986). It undergoes redox cycling *in vivo*, and is reduced by an electron donor and oxidised by an electron receptor such as oxygen to produce superoxide radicals (Bus and Gibson 1984). Thermal stress and ageing both result in cellular degeneration and an increasing amount of altered and nonfunctioning proteins (Lithgow et al. 1994). Acute thermal stress released by the temperature up-shift to 37 °C leads to the rapid death of *C. elegans*. The RO 90-7501-mediated longevity of *C. elegans* is presumably correlated with an increase in resistance to oxidative and thermal stress. Treatment with punicalagin did not result in an increase in tolerance to oxidative or thermal stress (data not shown). In this particular case, resistance to oxidative or thermal stress is not the reason or the consequence of lifespan extension. Punicalagin seems to induce longevity of *C. elegans* by differing mechanisms. Stress has multiple causes; thus, punicalagin may induce resistance against other stressors, such as UV, pathogens, or oxygen (Cypser and Johnson 2002). This was not however, tested in this work.

These findings initiated to analyse the underlying mechanisms of punicalagin- and RO 90-7501-induced lifespan extension. Therefore, metabolic parameters,

such as respiration were determined. After 6 h of exposure to punicalagin, oxygen consumption of nematodes was significantly inhibited, whereas after incubation for 5 d, respiration was similar to that of the untreated control nematodes. The respiration of the nematodes seemed to be temporarily blocked or inhibited after 6h and compensated to levels of the control after 5 d. Interestingly, the oxygen consumption of RO 90-7501-treated nematodes was also inhibited after short-term exposure (6h) to the compound. At 24 h, 48h and 5d after exposure, respiration significantly increased. Thus, the oxygen consumption was not only compensated, an induction of mitochondrial metabolism accompanied by an induced respiration occurred at later time points under RO 90-7501 treatment. Respiration is a metabolic process, where the organism obtains energy by reacting oxygen with glucose, which partly occurs in the mitochondria. That means an induction of mitochondrial metabolism can lead to higher oxygen consumption and can result in an increased formation of ATP.

The temporarily inhibited respiration of the nematodes exposed to punicalagin and RO 90-7501 led us to ask for the events happening within the mitochondria that can inhibit the respiration. For rotenone the inhibition of respiration is caused by an inhibition in complex I activity of the ETC (Chance and Hollunger 1963; Fato et al. 2009). The complex I enzyme (NADH:ubiquinone oxidoreductase) is the entry point of the mitochondrial ETC. It oxidises NADH to NAD^+ , transferring electrons to a lipid soluble electron carrier, ubiquinone or coenzyme Q. FMN is the direct electron acceptor of NADH, and electrons are transferred to the iron-sulphur clusters. Complex I is one of the main sites of production of harmful superoxide radicals (Fato et al. 2009). To test whether punicalagin and RO 90-7501 also inhibit complex I of the ETC, its activity was assayed in isolated mouse mitochondria. After treatment with punicalagin and RO 90-7501, complex I activity was significantly inhibited. To verify that the temporary decrease in respiration of *C. elegans* is caused by inhibition of complex I by both compounds, another respiration measurement was performed by using isolated mitochondria from rats. The respiration of the mitochondria was monitored utilizing NAD-dependent (glutamate/malate) substrates. Both energetic substrates are specific for complex I

(Gyulkhandanyan and Pennefather 2004). The modulation of oxygen consumption was analysed with and without application of punicalagin and RO 90-750. Both punicalagin and RO 90-7501 significantly inhibited oxygen consumption of isolated rat mitochondria. The inhibition occurred at complex I because the NAD-dependent substrates glutamate and malate are primarily utilized at complex I. Deductively, punicalagin and RO 90-7501 can temporarily decrease the respiration of *C. elegans* via the inhibition of complex I of the ETC.

The ATP content of the nematodes is another parameter to determine metabolic changes. ATP is generated during aerobic oxidative phosphorylation. The mitochondria produce most cellular ATP via the ETC (Wang et al. 2013). In fact, a disruption of the electron transport over the chain because of the inhibition of complex I should lead to a decrease in ATP formation. As expected, short-term exposure with punicalagin for 6 h significantly reduced ATP content of *C. elegans*. This short-term decrease was compensated by a significantly increased generation of ATP after long-term exposure (5 d). RO 90-7501 also significantly reduced ATP content after 6 h, whereas the 24 h, 48 h and 5 d treatments resulted in no significant changes; the decrease in ATP was compensated to the levels of the untreated nematodes. The temporarily decreased ATP levels can be seen as a result of the short-term impairment of the respiration of *C. elegans*. The lifespan extension initiated by inhibition of mitochondrial function, which is characterized by reduced oxygen consumption and lowered energy levels in *C. elegans*, has been reported earlier by Feng et al. (Feng et al. 2001).

To test whether glucose metabolism in nematodes is also influenced by the inhibition of the complex I, the glucose oxidation was determined by using labeled [^{14}C] D-glucose. Punicalagin significantly reduced glucose oxidation after short-term treatment (6h), reflected by an impaired production of ^{14}C -labeled carbon dioxide. The inhibition of glucose oxidation by punicalagin can be seen as the reason for the short-term impairment of respiration and decreased ATP levels; more likely it is the consequence of impaired respiration because of the inhibition of complex I. The enzyme of complex I oxidises NADH to NAD^+ . By blocking complex I, levels of NADH can increase, leading to a lack

of NAD⁺ in the citric acid cycle (Liu et al. 2002). The accumulation of acetyl coenzyme A results in negative feedback to the glycolysis and glucose metabolism is disrupted.

This metabolic stress initiated by these changes in energy and glucose metabolism and induced by punicalagin could represent the condition of CR known to be one important intervention against ageing and age-related diseases, and thus promoting longevity in *C. elegans* (Lakowski and Hekimi 1998). Caloric restriction is also linked to decreased body fat and food uptake (Liao et al. 2011; Schulz et al. 2007). In this work no significant changes in fat content and pumping rate of *C. elegans* after long-term treatment with punicalagin could be determined, suggesting that CR may not be the potential mechanism behind the punicalagin-mediated longevity. The nematodes did not lose body fat after 5 d of treatment with punicalagin. Permanent disruption of glucose metabolism under conditions of CR would probably lead to increased β -oxidation of fatty acids to maintain energy metabolism of the nematodes. Furthermore, the pumping rate of the worms did not change after treatment with punicalagin for 10 d. This reflects on the one hand that the nematodes did not refuse the uptake of punicalagin-containing food and on the other hand that no general decrease in food uptake occurred, which was reported to promote longevity in the case of CR (Lakowski and Hekimi 1998). It can be assumed that besides the temporary inhibition of glucose oxidation, CR is not the superficial mechanism behind the punicalagin-mediated longevity of *C. elegans*.

According to the deprivation of ATP and the arising changes in energy levels after short-term incubation of *C. elegans* with punicalagin and RO 90-7501, a low energy-sensing kinase, the AMPK/AAK-2 was examined for their role in the lifespan extension afforded by punicalagin and RO 90-7501. AAK-2 couples lifespan to information about energy levels and insulin-like signals in *C. elegans* (Apfeld et al. 2004). AMPK (AMP-activated kinase) is known to mediate cellular and metabolic responses to conditions that lower energy levels. This heterotrimeric serine/threonine kinase is activated by AMP and ADP and therefore maintains the energy balance by activating metabolic pathways that generate ATP and inactivating pathways that consume ATP (Kahn et al. 2005).

The kinase can regulate autophagy, mitochondrial biogenesis and food uptake (Zong et al. 2002; Minokoshi et al. 2004). *Caenorhabditis elegans* AMPK α subunit AAK-2 is necessary for CR-induced longevity (Greer et al. 2007). The AMP:ATP ratio changes in response to environmental stress, such as thermal stress, metabolic stress, or mitochondrial poisoning, or even during normal ageing, leading to AAK-2-mediated longevity in *C. elegans* (Apfeld et al. 2004). Conversely, AAK-2 and the transcription factor FoxO/DAF-16 are not necessarily needed for longevity of *eat-2* mutants (disruption of the pharyngeal function leading to partial starvation) of *C. elegans* (Lakowski and Hekimi 1998). In this work, the lifespan of AAK-2-deficient mutants of *C. elegans* was not extended upon exposure to punicalagin and RO 90-7501; consequently, the longevity of *C. elegans* afforded by punicalagin and RO 90-7501 is AAK-2-mediated. The activation of AAK-2, followed by an induction of metabolic pathways that generate ATP and induce mitochondrial biogenesis, and therefore reactivation of mitochondrial metabolism, should be the reason for the compensated (RO 90-7501) or even increased ATP levels (punicalagin), as well as the compensated (punicalagin) or even improved respiration rate (RO 90-7501) after long-term exposure to both compounds. Drugs, which inhibit the electron transfer through the ETC, were reported to increase lifespan of rodents by increasing the AMP:ATP ratio and activating AMPK (Owen et al. 2000; Anisimov et al. 2003). In *C. elegans*, an increased AMP:ATP ratio resulting from mitochondrial dysfunction activated AAK-2 leading to lifespan extension (Curtis et al. 2006). A possible mechanism by which AMPK can extend lifespan is by the phosphorylation of a large number of substrates, resulting in changes in feeding, metabolism, and transcription (Hardie 2004). AAK-2 may also regulate the transcription or activation of FoxO/DAF-16 target genes, which are involved in stress response or extracellular signaling of *C. elegans* (Lee et al. 2003). Thus, Punicalagin- and RO 90-7501-induced lifespan extension is mediated by AAK-2 due to an increased AMP:ATP ratio, resulting from a disruption of the electron transport through the ETC, followed by mitochondrial dysfunction and the occurrence of metabolic stress.

Based on the findings that punicalagin and RO 90-7501 both inhibit the complex I of the ETC, it was of interest to examine their influence on ROS levels of

C. elegans. Mitochondria are a major source of reduced oxygen derivatives, such as superoxide anion O_2^- , hydrogen peroxide H_2O_2 , and the hydroxyl radical OH^\cdot . Besides normal physiological processes, ROS can also be formed by enzyme systems or by exposure to ionizing and UV radiation, as well as during the metabolism of drugs and xenobiotics (Birben et al. 2012). Mitochondrial-derived ROS (mtROS) are associated with oxidative damage by altering the DNA, proteins and lipids, leading to functional decline, which is one typical characteristic of ageing (Lee and Wei 2007). There is also evidence that ROS take over the role of signal molecules (Koopman et al. 2010). The main source for O_2^- are the complex I and III of the ETC. Superoxide anions are rapidly dismutated into hydrogen peroxide by the mitochondrial SOD (Gyulkhandanyan and Pennefather 2004). In this work, two methods were used to determine the formation of mtROS in *C. elegans* exposed to punicalagin and RO 90-7501: the cell-permeant Mitotracker Red dye, which accumulates in active mitochondria and fluoresces when oxidised by mtROS (Li and Xing 2011), and the Amplex Red dye, which reacts with hydrogen peroxide in the supernatant of worms to produce highly fluorescent resorufin, which is catalysed by the horseradish peroxidase (Dikalov et al. 2007). Superoxide anions are able to cross through anion channels into the extramitochondrial space, thus, not only the mitochondrial SOD, but also the SOD located in the intermembrane region can convert it to H_2O_2 (Han et al. 2003). Hydrogen peroxide itself can penetrate through the mitochondrial membranes and, besides O_2^- , is a good indicator of oxidative stress arising from modulations of the ETC (Gyulkhandanyan and Pennefather 2004). After short-term incubation of the nematodes with punicalagin, a significant increase in mtROS could be determined by the use of Mitotracker Red. This increase was no longer evident after long-term incubation with punicalagin for 5 d. To verify these results, the amount of H_2O_2 formed under treatment with punicalagin was estimated. Confirming the findings of the Mitotracker Red assay, Amplex Red also showed a significant increase in H_2O_2 production after short-term incubation and H_2O_2 levels similar to the control after 5 d. Similarly, in the RO 90-7501 treatments, a significant increase in the formation of mtROS could be detected after 6 h, which was compensated after 24h and 48h, finally leading to a significant decrease after 5d. Interestingly, punicalagin with its large polyphenolic structure is supposed to have high

antioxidant capacity (Rosenblat et al. 2013; Sestili et al. 2007). In this work, however, punicalagin seems to transiently increase the formation of mtROS after short-term exposure. It is possible, that this prooxidant capacity of punicalagin as a large polyphenol can occur due to the artifactual generation of H_2O_2 in the medium, but this was disproved by Carreras et al. (2012). He showed that addition of catalase to the medium did not affect the effects of punicalagin on cell viability which suggested, that punicalagin did not generate the superoxide radical (as the first step in the formation of H_2O_2) extracellularly (Carreras et al. 2012). Therefore, punicalagin transiently increases the mtROS formation because of the inhibition of complex I. The increasing cellular O_2^- levels were also reported for the complex I inhibitor rotenone (Koopman et al. 2005). Increased O_2^- levels can result in increased levels of H_2O_2 , which is reflected by the Amplex Red measurement for punicalagin. The compensated or even significantly decreased ROS levels after long-term treatment with punicalagin and RO 90-7501 could be attributed to a possible induction of antioxidant enzymes and adaptive response being reported to result in stress resistance and longevity (Guan et al. 2010). This may also indicate mitochondrial hormesis, whereby a transient increase in mtROS formation causes an increase in ROS-defense mechanisms resulting in stress resistance and longevity (Ristow and Schmeisser 2011).

Contrary to the older thinking that ROS are particularly harmful and restrict lifespan (Sohal and Allen 1985), recent findings suggest that oxidative stress can induce longevity genes and an adaptive response (Sukata et al. 2002). Supplementation with antioxidants scavenging ROS diminishes the effect of dietary restriction or exercise on organisms (Ristow et al. 2009; Schulz et al. 2007; Gomez-Cabrera et al. 2008). To figure out whether ROS play a major role in the punicalagin- and RO 90-7501-mediated longevity in this work, and if supplementation with an antioxidant is able to abolish the effects as being reported, lifespan analyses with wild type nematodes exposed to punicalagin and RO 90-7501 were repeated with additional supplementation of an antioxidant (BHA). Punicalagin significantly extended the lifespan of wild type nematodes, whereas BHA alone had no effect on lifespan. The combination of punicalagin and the antioxidant also had no effect on lifespan; the antioxidant

completely abolished the lifespan-extending effect of punicalagin. The same could be found for RO 90-7501, which significantly increased lifespan, whereas the combination of the compound and the antioxidant showed no effect on lifespan of *C. elegans*. It can be assumed that for both compounds, ROS playing a major role in modulating pathways that induce stress defense and longevity of *C. elegans*, because of after scavenging ROS by the antioxidant BHA, the lifespan-extending effect disappeared. Supplementation with an antioxidant completely cancelled the effects induced by both compounds, which is consistent with recent findings. ROS may be seen as potential hormetins, where low-doses can have improving, and high-doses harmful effects. It should be discussed whether antioxidant supplements are really useful and promote health as has long been reported.

Because punicalagin and RO 90-7501 both induce a transient increase of mtROS and thus generate a condition of oxidative stress in *C. elegans*, the p38 mitogen-activated protein kinase (MAPK), which regulates the oxidative stress response and serves as a transducer of extracellular stimuli leading to adaptive responses (Inoue et al. 2005), was investigated for its role in stress resistance and longevity of *C. elegans* afforded by punicalagin and RO 90-7501. Oxidative stress can activate p38 MAPK (Chang and Karin 2001). Although *C. elegans* has two p38 MAPK orthologues, PMK-1 and PMK-2 (Berman et al. 2001), only PMK-1-deficient mutants are immunodeficient (Troemel et al. 2006). The transcription factor SKN-1 functions as a downstream target of PMK-1 and is activated via phosphorylation, resulting in an upregulation of antioxidant enzymes and enhanced resistance to stress in *C. elegans* (Inoue et al. 2005). Both compounds were tested for their influence on lifespan in PMK-1-deficient mutants of *C. elegans*. The significant lifespan-extending effects of punicalagin and RO 90-7501 were abolished in *pmk-1* KO mutants. It can be assumed that PMK-1 is involved in signaling pathways leading to longevity of *C. elegans* mediated by punicalagin and RO 90-7501.

Oxidative stress, which is induced by the transient increase of mtROS after short-term incubation with both compounds, leads to the activation of PMK-1 and possibly to the direct phosphorylation of Nrf2 [NF-E2 (nuclear factor-E2)-related factor 2]/SKN-1, resulting in adaptive response actions. Subsequently,

the role of SKN-1 was evaluated in the signaling pathway activated by the oxidative stress induced by punicalagin and RO 90-7501. Besides mitochondrial dysfunction, proteosomal dysfunction can also activate SKN-1 in *C. elegans* (Kahn et al. 2008). The *skn-1* gene of *C. elegans* encodes three protein isoforms, SKN-1a, b and c. SKN-1b is expressed in the ASI neurons but is not detectable in the gut and was reported to mediate lifespan extension in response to CR (Bishop and Guarente 2007), whereas SKN-1a and c are expressed in the intestine, but not detectably in the ASI neurons. SKN-1 is required for oxidative stress resistance and contributes to longevity by reduced insulin/IGF1 signaling (Tullet et al. 2008). Under conditions of oxidative stress, SKN-1 translocates from the cytosol to the nucleus of intestinal cells, where it induces the expression of genes involved in oxidative stress response such as superoxide dismutases, catalases, and glutathione S-transferases (Park et al. 2009), a process that can be induced by PMK-1 (Inoue et al. 2005). SKN-1 also modulates lifespan of *C. elegans* under normal conditions, mutants lacking SKN-1 have a shortened lifespan and impaired stress resistance (An and Blackwell 2003). To test if SKN-1 is involved in the lifespan extension induced by punicalagin and RO 90-7501 and possibly acts downstream of PMK-1 to induce adaptive responses, a global knock-out of *skn-1* (zu135) was used to perform lifespan analyses with application of punicalagin or RO 90-7501.

The significant lifespan-extending effect of both compounds was completely abolished in a global *skn-1* KO mutant of *C. elegans*. It can be assessed that the longevity afforded by punicalagin and RO 90-7501 is mediated by the transcription factor SKN-1. Because of *C. elegans* SKN-1 has three isoforms expressed in different parts of the nematode, it was of interest to determine which site(s) of SKN-1 expression were the most important for the punicalagin- and RO 90-7501-induced effects. Therefore, another *skn-1* knock-out mutant of *C. elegans* was tested in lifespan analyses, the *skn-1* (zu67), a KO for only SKN-1 expressed in the intestine. Treatments with both compounds did not result in a significant increase in lifespan of *skn-1* (zu67) KO mutants. It can be concluded that SKN-1 expressed in the intestine is involved in the lifespan extension induced by punicalagin and RO 90-7501. Because no knock-out mutant for SKN-1 expressed in the ASI neurons was available, another mutant strain using an integrated transgene to restore the SKN-1 function in the

intestine was used to determine the role of SKN-1 expressed in the neurons (Powolny et al. 2011). Whereas treatment with punicalagin significantly extended lifespan in the *skn-1* (LG357) KO mutant of *C. elegans*, the effect of RO 90-7501 was completely abolished in this global KO mutant with the restored SKN-1 function in the intestine. In summary, the lifespan extension of *C. elegans* afforded by punicalagin is SKN-1-mediated, but only the isoform expressed in the intestine is involved in the signaling pathways leading to punicalagin-induced longevity. In the case of RO 90-7501, both, SKN-1 expressed in the ASI neurons and intestine mediate lifespan extension.

Another important transcription factor, FoxO/DAF-16, functions in the *C. elegans* insulin/IGF1 signaling pathway and also promotes longevity (Hsu et al. 2003). Inhibition of DAF-16 activity shortens the lifespan of *C. elegans* wild type nematodes, whereas increased activity promotes longevity (Kenyon et al. 1993). DAF-16 is important for many processes including development, stress resistance, thermotolerance, pathogen resistance, metabolism, and autophagy (Baugh and Sternberg 2006). Besides the transcriptional targets including stress, heat, and metabolic response most of the genes strongly regulated by DAF-16 influence ageing of *C. elegans* (Murphy et al. 2003). Therefore, DAF-16 functions in parallel to the transcription factor SKN-1 to regulate adaptive responses to stress and promote longevity by translocation from cytoplasm to nucleus under conditions of stress. The ability of AAK-2 to mediate lifespan extension of *C. elegans* depends on the presence of intact DAF-16 (Greer et al. 2007). To determine whether DAF-16 is also involved in the longevity induced by punicalagin and RO 90-7501, DAF-16-deficient mutants of *C. elegans* were used for lifespan analyses. In the *daf-16* KO mutant, the significant lifespan-extending effect of punicalagin was still evident, although the effect was not as high as detected in wild type nematodes. The longevity induced by punicalagin is not or only partly mediated by DAF-16. This has also been reported for resveratrol, which still extends the lifespan of DAF-16-deficient mutants of *C. elegans* (Greer and Brunet 2009). Punicalagin extends lifespan by an AAK-2-dependent but DAF-16-independent pathway, although activation of AMPK leads to the induction of FoxO in mammals (Greer et al. 2007). Therefore, activation of AMPK is not always coupled to that of FoxO, although

the ability of AMPK to mediate lifespan extension depends on the presence of intact FoxO (Apfeld et al. 2004; Greer et al. 2007). The effect on lifespan initiated by RO 90-7501 was completely abolished in DAF-16-deficient mutants. For this compound, it can be assumed that the transcription factor DAF-16 is involved in the lifespan extension via direct regulation by AAK-2 or in parallel to AAK-2.

As the transcription factors SKN-1 and partly DAF-16 are involved in the lifespan extension and stress resistance afforded by punicalagin and RO 90-7501, two important endogenous antioxidant enzyme systems, SOD and CTL, playing an important role in the elimination of ROS, which is generated under normal as well as under conditions of stress, were determined. SOD catalytically removes the superoxide radical O_2^- . In *C. elegans* five SOD-encoding genes have been identified; all SOD isoenzymes help to control normal ageing and longevity (Yanase et al. 2009). *Sod-3* and *sod-2* encode a mitochondrial iron/manganese SOD, which might defend against oxidative stress and promote normal lifespan (Wang et al. 2012 225; Van Raamsdonk and Hekimi 2009). *Sod-5* and *sod-1* encode two cytoplasmic copper/zinc SODs, which are known to protect cells from oxidative damage. *Sod-4* encodes an extracellular Cu^{2+}/Zn^{2+} SOD that is another one of five *C. elegans* SOD enzymes (Panowski et al. 2007). The insulin/IGF1 signaling pathway is involved in the regulation of SODs via DAF-16 (Panowski et al. 2007). One pathway that is acting in parallel to the insulin/IGF1 signaling is the p38 MAPK pathway, which participates in the stress-mediated translocation of DAF-16 to the nuclei and thus also induces the activity of SODs in *C. elegans* (Kondo et al. 2005). This is supported by the finding that the transcription factor SKN-1, which also can be activated by PMK-1, itself induces genes such as *sod-1* in response to oxidative stress (Park et al. 2009).

Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al. 2004). The cytosolic *ctl-1* and *ctl-3* encode two of three *C. elegans* enzymes, exhibiting catalase activity, whereas the peroxisomal *ctl-2* encodes the third *C. elegans* enzyme, which exhibits catalase and peroxidase activity. Besides *sod-3*, which is regulated by the transcription factor DAF-16

(Murphy et al. 2003), and *sod-1*, which is regulated by SKN-1; *ctl-2* and *ctl-3* are target genes induced by both DAF-16 and SKN-1 (Park et al. 2009).

In this work, the activity of both enzymes, SOD and CTL was determined in *C. elegans* after exposure to punicalagin and RO 90-7501. Long-term incubation with punicalagin (5 d) resulted in no difference in the activity of SOD compared to untreated nematodes, whereas the activity of CTL was significantly increased after 5 d of application. RO 90-7501 significantly increased the activity of both enzymes after a 24 h exposure. These results reflect that oxidative stress, which is induced by the inhibition of complex I of the ETC by both compounds and the therefore transient elevated release of mtROS, leads to an activation of antioxidant enzymes via stress response pathways, resulting in increased stress defense and longevity of *C. elegans*. In the case of RO 90-7501, both antioxidant enzymes are already induced after 24 h, leading to the significant decrease of ROS after 5d. Both the transcription factors DAF-16 and SKN-1, which are activated by oxidative stress, seem to regulate the expression of the antioxidant enzymes and the resulting stress resistance and longevity of *C. elegans*. Treatment with punicalagin results in no activation of SOD, whereas CTL is significantly induced after 5 d. This may not lead to a significant decrease of ROS after 5d, but to the compensated levels after the transient increase at 6 h. The transcription factor DAF-16 is not involved in the lifespan extension afforded by punicalagin. DAF-16 may primarily regulate SOD activity, and SKN-1 may be the major transcription factor for the CTL activation. This would explain that the activity of CTL is significantly induced by punicalagin after 5d, although SOD activity remains unaffected. The punicalagin-mediated effects seem to be PMK-1-dependent but DAF-16-independent, whereas the effects of RO 90-7501 on *C. elegans* are PMK-1- and DAF-16- dependent.

For punicalagin, no increased resistance against thermal or oxidative stress could be detected. This raised the question of why the nematodes have an increased lifespan following an induced antioxidant capacity by upregulation of different signaling pathways and an improved mobility. Punicalagin may not increase the resistance against oxidative and thermal stress, but against other stressors. One important part in declining the ageing process may be the capacity to maintain protein homeostasis and to reduce proteotoxicity induced

by increasing amounts of unfolded and misfolded proteins. Besides that, several compounds (including RO 90-7501) have protein-aggregate-binding capacities (Bohrmann et al. 2000) and can reduce age-dependent misfolding and accumulation of proteins. A *C. elegans* model of human proteotoxic disease was used to determine the possible influence of punicalagin and RO 90-7501 on the formation and accumulation of protein aggregates. The transgenic strain CL 4176 [*smg-1(cc546)* I; *dvIs27* X] expresses muscle-specific A β ₁₋₄₂ with raising the temperature to 25°C. The control CL 802 [*smg-1(cc546)* I; *rol-6(su1006)* II] also contains this transgene, but does not undergo paralysis with temperature up-shift (Link et al. 2003). Transgenic nematodes CL4176 showed a significant decrease in formation of protein aggregates in muscle cells after exposure to punicalagin or RO 90-7501, while the transgenic control strain showed no formation of aggregates as expected.

This proves the identified inhibitory effect of RO 90-7501 on the formation of A β ₁₋₄₂ fibrils (Bohrmann et al. 2000). For only punicalagin, none of these effects have been reported, but pomegranate extract seemed to attenuate A β plaque deposition in a transgenic mouse model of Alzheimer's disease (Rojanathammanee et al. 2013). This may represent one possible explanation for the decline of ageing and the induced longevity afforded by punicalagin. The Nrf2/SKN-1 pathway induces resistance against oxidative stress (An and Blackwell 2003), and may also play a general role in modulating A β accumulation in *C. elegans* as described for caffeine (Dostal et al. 2010), and in an APP/PS1 transgenic mouse model (Kanninen et al. 2008). The activation of the transcription factor SKN-1, as we described for punicalagin and RO 90-7501 may be protective in a range of neurodegenerative conditions (Chen et al. 2009). Misfolded proteins could also act through SKN-1 because proteosomal dysfunction activates SKN-1 in *C. elegans* (Kahn et al. 2008). The protective effects of punicalagin and RO 90-7501 against paralysis induced by A β ₁₋₄₂ expression are potentially SKN-1-mediated. Besides SKN-1, DAF-16 also regulates a large number of genes involved in lifespan control, stress tolerance, and protein misfolding suppression (Ogg et al. 1997; Lin et al. 1997). The DAF-16 transcription factor, which does not play a role in punicalagin-mediated lifespan extension, may be involved in the protective effects against proteotoxicity in *C. elegans* under exposure to RO 90-7501.

The heat shock transcription factor HSF-1 (homologous to mammalian HSF1) also acts in parallel to DAF-16 to activate heat shock genes in response to heat and other forms of stress in *C. elegans* (Hsu et al. 2003). HSF-1 induces activation of various heat shock genes or chaperones that are involved in maintaining and re-establishing proteostasis by rescuing misfolded proteins (Rodriguez et al.). HSF-1 is also involved in ageing; reducing the activity of HSF-1 in *C. elegans* leads to a shortened lifespan, following a reduced chaperone expression (Garigan et al. 2002), whereas overexpression promotes longevity and delays age-related protein misfolding and proteotoxicity (Hsu et al. 2003). Heat shock proteins, which can be activated by DAF-16 and HSF-1, are known to form oligomers which bind to unfolded proteins and prevent them from aggregating (Horwitz 1992). Additionally, heat shock proteins 70 and 90 inhibit early stages of A β ₁₋₄₂ aggregation *in vitro* (Evans et al. 2006). Because punicalagin and RO 90-7501 can slow protein aggregation of a *C. elegans* model of human proteotoxic disease, the role of HSF-1 in mediating protein homeostasis and longevity of *C. elegans* was investigated. Punicalagin significantly shortened the lifespan of an HSF-1-deficient mutant of *C. elegans*, whereas the lifespan extension afforded by RO 90-7501 was completely abolished in this mutant. Both results suggest that the lifespan-extending effect mediated by both compounds is HSF-1-dependent. Because of the toxic and lifespan-shortening effect of punicalagin in the *hsf-1* KO mutant, it can be speculated that the lifespan-extending effect is primarily mediated by HSF-1; the effect is SKN-1- and HSF-1-dependent but DAF-16-independent, while the effect of RO 90-7501 is dependent of SKN-1, HSF-1 and DAF-16, directing the focus to the enhanced proteostasis and therefore decelerated ageing process in the case of punicalagin.

To verify the role of HSF-1 in the longevity induced by punicalagin and RO 90-7501, one direct target of HSF-1, AIP-1 (homologous to mammalian AIRAP) was investigated for its role (Calabrese 2005). AIRAP (arsenic-inducible RNA-associated protein) was first identified as part of the response to arsenic (Sok et al. 2001). Arsenic produces ER stress, oxidative stress, and protein misfolding. Arsenic also triggers the expression of AIP-1 in nematodes via the transcription of SKN-1 (Stanhill et al. 2006). Thus a SKN-1-independent

activation of AIP-1 via HSF-1, or a SKN-1-dependent activation is possible. AIP-1 is required for normal lifespan of *C. elegans* (Yun et al. 2008), while increased expression of AIP-1 reduces A β accumulation and attenuates A β -induced paralysis in *C. elegans* (Hassan et al. 2009). AIP-1 acts by increasing the general protein turnover, stimulating the degradation of damaged proteins and buffering proteotoxicity (Yun et al. 2008). In this work AIP-1-deficient nematodes had a significantly shortened lifespan after exposure to punicalagin, whereas the lifespan-extending effect of RO 90-7501 was abolished in AIP-1-deficient mutants of *C. elegans*. AIP-1 is required for the lifespan-extending effect afforded by both compounds. Because punicalagin is not only toxic in HSF-1-deficient mutants, but also in AIP-1-deficient mutants, both seem to play a major role in the effects generated by punicalagin. It can also be speculated, that AIP-1 is mainly regulated by HSF-1, and that SKN-1 plays a minor role in regulation. The overload of protein misfolding may contribute to ageing. Thus, the transcription factor HSF-1 and the target gene *aip-1* also play an important role in the decline of ageing and age-related diseases. The proteasomal adaption to stress, which may be induced by punicalagin and RO 90-7501, links resistance against proteotoxicity to longevity of *C.elegans* (Yun et al. 2008).

After determining that punicalagin and RO 90-7501 can improve protein homeostasis and prevent against proteotoxicity, it was of interest to more closely investigate the pathways that regulate protein folding and thus protein homeostasis. Protein misfolding, accumulation and aggregation are increasing events during ageing, as well as under conditions of stress. Proteins are transformed from their native soluble form to insoluble fibrils or aggregates, which can accumulate. Under normal conditions, protein aggregates do not accumulate in cells because of control mechanisms (Brown and Naidoo 2012). One of those systems can be found in the endoplasmatic reticulum (UPR_{ER}), another one in the mitochondria (UPR_{MT}). Under stress conditions, which are triggered by misfolded proteins (Berridge 2002), the unfolded protein response (UPR) is activated, and adaptive mechanisms that regulate transcription and translation, chaperoning of unfolded proteins or the transport of misfolded

proteins through degradation, prevent the accumulation of unfolded/misfolded proteins (Ellgaard et al. 1999).

In contrast to heat shock response mediated by HSF1/HSF-1, which is predominantly a response to stress conditions in the cytosol of the cell, the UPR controls the secretory pathway (Mager and Ferreira 1993). Interestingly, there are targets of HSF1/HSF-1 which are also induced by the organelle-specific stress response pathways, e.g., the UPR_{ER}, as reported for the chaperone BIP/HSP-4 (Kohno et al. 1993). Otherwise, ER stress can induce heat shock response, although this is considerably less compared to the induction by heat stress (Liu and Chang 2008).

ER stress can be afforded by glucose/energy deprivation, redox changes, or hypoxia (Kaufman et al. 2002). To evaluate the role of the UPR_{ER} in the punicalagin- and RO 90-7501-mediated longevity of *C. elegans* and to learn more about the signaling pathway within the UPR_{ER}, knock-out mutants of *C. elegans* for the different parts of the signaling pathway were examined in lifespan analyses. The ER stress response consists of three transmembrane sensors: the inositol-requiring element 1 IRE1/IRE-1, the PKR-like ER kinase PERK/PEK-1, and the activating transcription factor 6 ATF6/ATF-6. IRE-1-, PEK-1- and ATF-6-deficient mutants of *C. elegans* were investigated for their involvement in the longevity of the nematodes afforded by punicalagin and RO 90-7501. In *atf-6* and *pek-1* KO mutants the lifespan-extending effect of punicalagin was completely abolished, whereas treatment with punicalagin significantly shortened the lifespan of an *ire-1* KO mutant. All three transmembrane sensors are involved in the lifespan extension afforded by punicalagin. Furthermore, it can be speculated that IRE-1 possibly has the major role in mediating the effect; punicalagin seems to have a toxic effect in *ire-1* KO mutants. Treatment with RO 90-7501 still results in a significant increase of lifespan in an *atf-6* KO mutant. ATF6 seems to play no role in the signaling pathway mediating the effect of RO 90-7501. While the lifespan-extending effect of that compound is abolished in an *ire-1* KO mutant, the lifespan is significantly shortened in the PEK-1-deficient mutant. Both transmembrane sensors mediate the lifespan-extending effect of RO 90-7501; the PEK-1 sensor seems to play the major role, and RO 90-7501 appears to be toxic in this mutant. All three sensors are maintained in an inactive state in the

ER membrane by binding to the ER chaperone BiP/HSP-4, which is a peptide-dependent ATPase and member of the heat shock 70 protein family (Brown and Naidoo 2012). By binding to unfolded/misfolded proteins, BiP dissociates from the sensors and one or more of the transducers are activated followed by activation of downstream events (Zhang and Kaufman 2006). The lifespan-extending effect of both punicalagin and RO 90-7501, was abolished in an HSP-4-deficient mutant. HSP-4 is involved in the longevity of the nematodes mediated by these compounds. The x-box-binding protein 1 XBP1/XBP-1 is a downstream target of IRE1/IRE-1. Activated IRE-1 leads to activation of XBP-1 via splicing (Brown and Naidoo 2012). The activated form acts as a transcription factor, which increases the expression of genes that are involved in ER homeostasis as well as in export and degradation of misfolded proteins (Yoshida et al. 2001). The lifespan-extending effect of both compounds was also abolished in an XBP-1-deficient mutant of *C. elegans*, thus, XBP-1 also mediates the effects generated by exposure to punicalagin and RO 90-7501. Taken together, it can be speculated that the effect of punicalagin is mediated by a signal pathway within the ER which is initiated by HSP-4. HSP-4 activates the transducers ATF-6, PEK-1 and IRE-1, although IRE-1 seems to have the major role in transducing the signal. IRE-1 itself activates XBP-1 via splicing, and the translation of genes involved in folding, protein maturation, and export and degradation of misfolded proteins increases. The effect of RO 90-7501 is mediated by the pathway also initiated by HSP-4, which activates only two of three transducers, PEK-1 and IRE-1, although PEK-1 seems to be most involved in transducing the signal.

One downstream target of PEK-1 is the translation initiation factor eIF2 α , which results in the enhanced translation of certain proteins, e.g., ATF4/ATF-5. The activating transcription factor 4 ATF4/ATF-5 initiates protective or apoptotic signaling after being activated (Brown and Naidoo 2012). Because PEK-1 seems to be most involved in the signal transduction following ER stress, a knock-out mutant for *atf-5* was also tested in lifespan analyses for its role in the RO 90-7501-mediated longevity. The effect of this compound was abolished in ATF-5-deficient mutants of *C. elegans*, which intensifies the idea that the PEK-1/ATF-5 axis is most involved in the RO 90-7501-mediated effects.

In addition all three axis function together and can compensate each other (Shen et al. 2001), which makes it complicate to determine the exact pathway involved. Besides the activation of antioxidant enzyme systems via DAF-16 and SKN-1 and the initiation of heat shock response via DAF-16 and HSF-1, the regulation of the UPR_{ER} seems to be another part for increased stress defense and decelerated ageing of *C. elegans* by punicalagin and RO 90-7501. Crosslinks between these pathways exist, thus PERK activation within the UPR_{ER} also up-regulates an antioxidant response through Nrf2 (Johnson et al. 2008). BiP/HSP-4 is also a direct target of HSF1/HSF-1, and is therefore not only up-regulated in cases of ER stress (Kohno et al. 1993). Additionally, DAF-16 synergises with XBP-1 to activate genes, which lead to enhanced ER stress resistance in *C. elegans* (Henis-Korenblit et al. 2010).

These results show that the UPR_{ER} of *C. elegans* is involved in longevity of *C. elegans* induced by punicalagin or RO 90-7501, although it is difficult to examine the exact signaling pathway. This up-regulation could also play a role in the deceleration of the ageing process and the induction of stress resistance through crosslinks of the signaling pathways. The impairment of the cellular machinery leading to an increase in unfolded/misfolded proteins and their accumulation is associated with ageing (Naidoo et al. 2008). Enhancing the activity of the UPR could lead to slowed ageing and reduced occurrence of age-related diseases. A functional and balanced ER is important for maintaining the integrity of the cell and for protecting the organism from age-related diseases such as heart disease, neurodegenerative disorders, and diabetes (Lin et al. 2008; Lindholm et al. 2006). Thus, genes that can regulate the ER stress response machinery are potential candidates for longevity genes. The induction of protective pathways seems to be important for longevity; inactivation of protection-regulatory genes was shown to shorten the longevity of *C. elegans* normally induced by decreased insulin/IGF1 signaling, disruption of mitochondrial function, or CR. These genes included *daf-16*, *skn-1* and *hsf-1*, but also genes from the UPR_{MT} (*hsp-6*, *hsp-60*), the UPR_{ER} (*hsp-4*, *ire-1*), ROS response (*sod-3*, *gst-4*), and xenobiotic detoxification (*gst-4*) (Shore et al. 2012).

Although mitochondria have their own machinery to respond to mitochondrial-specific stresses, which can be caused by the accumulation of

unfolded proteins, defects in respiration or altered mtROS metabolism, and is termed the mitochondrial unfolded protein response (UPR_{mt}), it can be argued, that attenuated translation in the cytosol and the ER can slow mitochondrial protein import. That can reduce the overall burden on the mitochondrial protein folding (Baker et al. 2012). Therefore, an up-regulation or enhanced maintenance of the UPR_{ER} and an even improved protein homeostasis also protects the mitochondria in case of oxidative stress.

5. Conclusion and perspectives

The results of this thesis suggest that punicalagin and RO 90-7501 are compounds that exert their action by targeting mitochondria. Both compounds were shown to inhibit complex I of the ETC which leads to mitochondrial dysfunction and results in a significant and transient decrease of respiration and ATP formation, as well as a transient increase in the release of mitochondrial-derived ROS. Cells can respond to impairments of mitochondrial function by activating signaling pathways that restore or even improve mitochondrial homeostasis and metabolism. This is leading to prevention against environmental stress and age-related diseases, culminating in a delay of ageing (Kirchman et al. 1999).

In *C. elegans*, punicalagin initially impairs mitochondrial function by inhibiting complex I of the ETC, resulting in an elevated and transient release of mtROS. Mitochondrial-derived ROS are not only involved in cell damaging mechanisms (Wei 1998), but in low-doses they also serve as important signal molecules that are involved in cellular signal transduction and regulation of a variety of processes, such as the induction of stress defense mechanisms as a secondary response to a stressful condition (Ristow and Schmeisser 2011). Following the inhibition of complex I of the ETC and the resulting short-term impairment of the respiration, glucose oxidation and ATP levels are significantly decreased. This short-term metabolic stress activates the energy-sensing kinase AAK-2, which in response promotes mitochondrial metabolism to restore homeostasis and subsequently increases respiration and ATP levels. The initial significant increase of mtROS released by complex I due to the inhibition by punicalagin is sensed by the stress kinase PMK-1 and activates the transcription factor SKN-1 (expressed in the intestine) and the heat shock factor HSF-1. This thereby causes an adaptive response following the oxidative stress to improve the stress defense and restore mitochondrial function, including up-regulation of the antioxidant enzyme CTL and improvement of protein homeostasis. This then terminates the initial ROS signal, in parallel leading to an extended lifespan of *C. elegans*, which is cancelled upon supplementation of an antioxidant (Figure 59).

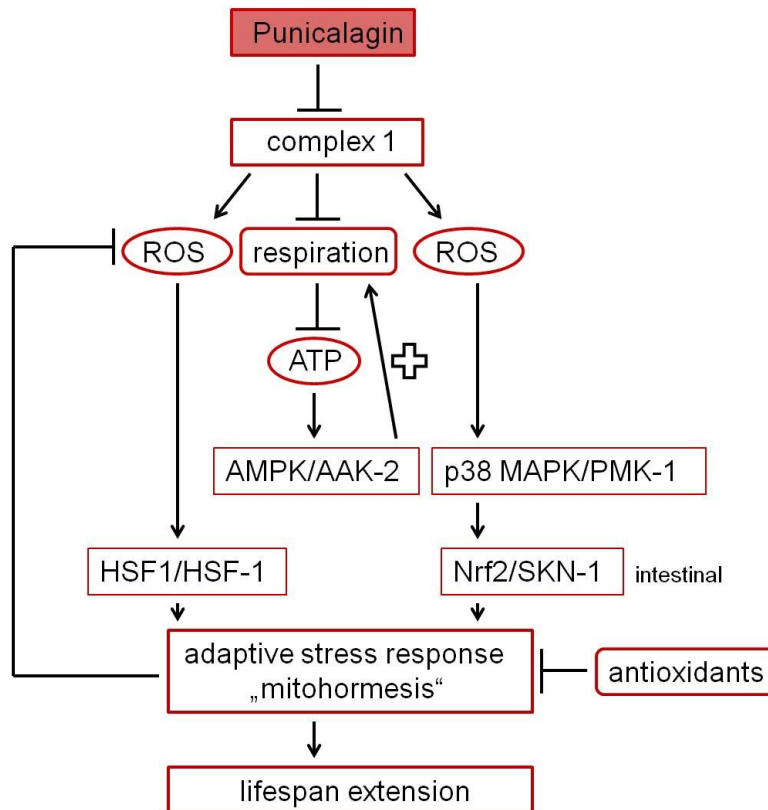


Figure 59: Mechanism of punicalagin actions in *C. elegans*

Impaired electron transport across the electron transport chain inhibits respiration and generates ROS as a mitochondrial second messenger culminating in extended lifespan: inhibition of complex I causes impaired respiration and decreased formation of ATP in *C. elegans*, which leads to an energy deficit that reactivates mitochondrial respiration in an AAK-2-dependent manner. Additionally, the transient ROS signal, which is sensed by PMK-1 and activates intestinal SKN-1 and heat shock factor HSF-1, causes an adaptive response to improve stress defense including the antioxidant enzyme CTL, or improvement of protein homeostasis and UPR, which then terminates the initial ROS signal, in parallel leading to an extended lifespan of *C. elegans*, which is cancelled upon supplementation of an antioxidant.

The inhibition of complex I initiated by RO 90-7501 also causes an impaired respiration and significantly decreased ATP formation after short-term exposure of 6 h, which leads to metabolic stress that promotes mitochondrial metabolism in an AAK-2-dependent manner and compensates for the short-term decreased respiration and ATP levels. Additionally, the transiently increased release of mtROS, which causes oxidative stress, is sensed by the stress kinase PMK-1, and activates the transcription factors DAF-16 and SKN-1 (expressed in the ASI neurons and the intestine) as well as the heat shock factor HSF-1, which again leads to signal pathways resulting in an adaptive response to improve the stress defense and restore mitochondrial function, including up-regulation of

antioxidant enzymes such as SOD and CTL, and improvement of protein homeostasis. This again terminates the initial ROS signal and in parallel, leads to an increased resistance against oxidative and thermal stress and an extended lifespan of *C. elegans*, which is cancelled upon supplementation of an antioxidant (Figure 60).

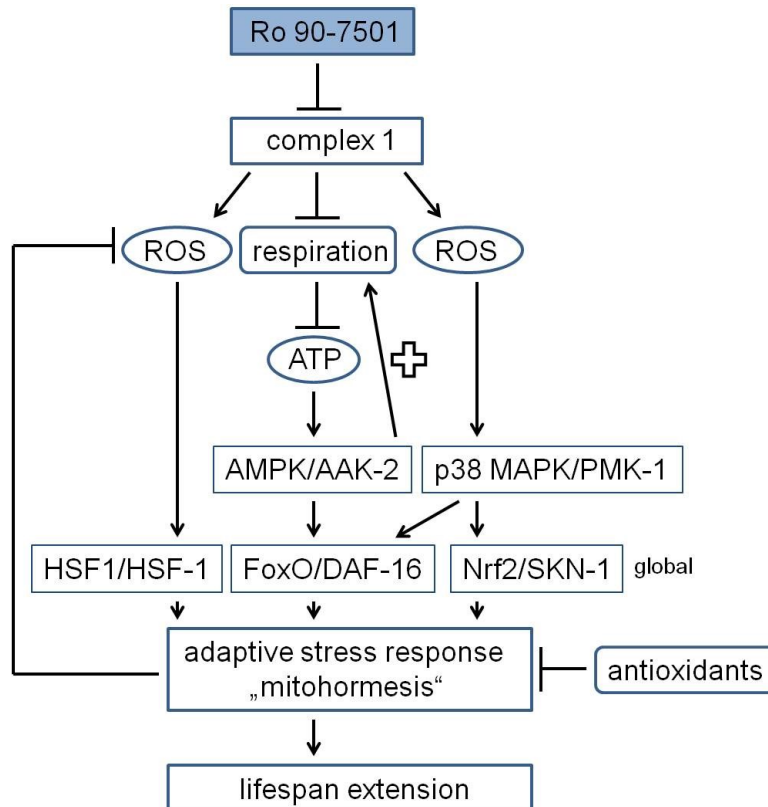


Figure 60: Mechanism of RO 90-7501 actions in *C. elegans*

Impaired electron transport across the electron transport chain inhibits respiration and generates ROS as a mitochondrial second messenger culminating in extended lifespan: inhibition of complex I causes impaired respiration and decreased formation of ATP in *C. elegans*, which leads to metabolic stress that reactivates mitochondrial respiration in an AAK-2-dependent manner. Additionally, the transient mtROS signal, which is sensed by PMK-1 and activates DAF-16, SKN-1 and HSF-1, causes an adaptive response to improve stress defense including antioxidant enzymes such as SOD or CTL, or improvement of protein homeostasis and UPR, which then terminates the initial ROS signal, in parallel leading to increased stress resistance and extended lifespan of *C. elegans*, which is cancelled upon supplementation of an antioxidant.

Taken together, both compounds can extend lifespan of *C. elegans* in a mitohormetic manner. Mitohormesis, which was first coined by Tapia (Tapia 2006), is the adaptive response to ROS-related stress emanating from the mitochondria leading to increased stress defense. Thus, besides CR and

exercise, which cause mitohormesis and lead to lifespan extension in several organisms (Schulz et al. 2007; Navarro et al. 2004), compounds that directly target the mitochondria, resulting in a transient release of mtROS, may also prevent ageing and age-related diseases in a mitohormetic manner.

A lifetime low-dose of oxidative stress could be beneficial by causing a secondary induction of defense mechanisms. This can protect the organism from high ROS exposure and possibly delay ageing. Ageing is a process in which changes to molecular and cellular structures accumulate. The stress response decreases, and homeostatic imbalance increases. By improving the mitochondrial metabolism and inducing stress-defense mechanisms in a mitohormetic manner, it may be possible to decelerate ageing and not only extend the lifespan, but also improve the quality of life, as shown by the increase of locomotion of *C. elegans* under exposure to punicalagin and RO 90-7501. Furthermore, prevention against age-related diseases is feasible. Punicalagin and RO 90-7501 can reduce the formation of muscle-specific A β ₁₋₄₂ in a *C. elegans* model of human proteotoxic disease.

The results presented in this work suggest a possible health-beneficial and age-decelerating effect of punicalagin and RO 90-7501. Although both compounds extend lifespan of *C. elegans* in a mitohormetic manner, it would be worthwhile to evaluate the effects in rodents and potentially in humans in regards to the prevention of ageing and age-related diseases.

Moreover, a deeper look into protein homeostasis of *C. elegans* under exposure to punicalagin and RO 90-7501, including the UPR_{MT}, the proteasome and the interaction between all these pathways, would be of interest to examine the possible protective effect against neurodegenerative diseases such as Alzheimer's disease.

Moreover, additional genes are likely involved in the punicalagin- and RO 90-7501-mediated longevity of *C. elegans*, which have not been studied in the present thesis. Therefore, analysis of gene expression also would be of special interest.

Appendices

I Strains used

Table 1: Strains used

strain	provided by:
<i>E. coli</i> (OP50, OP50i)	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> (wild type, N2)	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>aak-2</i> (ok524)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>pmk-1</i> (km25)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>skn-1</i> (zu135)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>skn-1</i> (zu67)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>skn-1</i> (zu135) IV/nT1[qIs51] (IV;V); gels10]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>daf-16</i> (mu86)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>hsf-1</i> (sy441)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>aip-1</i> (ok537)]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>smg-1</i> (cc546) I; dvIs27 X]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>smg-1</i> (cc546) I; rol-6(su1006) II]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA

<i>C. elegans</i> [<i>ire-1(ok799)</i>]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>pek-1(ok275)</i>]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>atf-6(ok551)</i>]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>xbp-1(zc12)</i>]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>hsp-4(gk514)</i>]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA
<i>C. elegans</i> [<i>atf-5(ok576)</i>]	Caenorhabditis Genetics Center (CGC) University of Minnesota, USA

II Statistical analyses of lifespan assays

Table 2: Results and statistical analyses of lifespan assays modulated by punicalagin

Strain, Substance, Solvent	Maximum Life Span [d] [+/- SEM] ¹	Mean Life Span [d] [+/- SEM]	P-value (vs. control, see footnotes)	Number of experiments [n]	Number of nematodes [n]
N2 DMSO	24.7 ± 0.7	22.41 ± 0.2		3	271
N2 PUNIC/DMSO	26.7 ± 0.7	24.07 ± 0.3	< 0.0001 ^a	3	283
N2 DMSO	24.0 ± 0.0	22.29 ± 0.1		3	306
N2 BHA/DMSO	24.0 ± 0.0	22.60 ± 0.2	n.s. ^a	3	339
N2 PUNIC/BHA/DMSO	24.0 ± 0.0	22.35 ± 0.1	n.s. ^a	3	293
N2 PUNIC/DMSO	26.0 ± 0.0	23.53 ± 0.2	< 0.0001 ^a	3	291
N2 DMSO	29.0 ± 1.0	26.94 ± 0.3		3	410
N2 EA/DMSO	31.0 ± 0.0	27.45 ± 0.2	n.s. ^a	3	388
aak-2(ok524) DMSO	25.3 ± 0.7	22.46 ± 0.6		3	359
aak-2(ok524) PUNIC/DMSO	24.0 ± 0.0	22.36 ± 0.2	n.s. ^c	3	374
pmk-1(km25) DMSO	24.0 ± 0.0	21.90 ± 0.0		3	139
pmk-1(km25) PUNIC/DMSO	24.0 ± 0.0	21.83 ± 0.2	n.s. ^d	3	139
skn-1(zu135) DMSO	22.0 ± 0.0	19.76 ± 0.2		3	372
skn-1(zu135) PUNIC/DMSO	22.0 ± 0.0	19.74 ± 0.1	n.s. ^e	3	267
skn-1(zu67) DMSO	19.0 ± 0.0	17.96 ± 0.0		3	293
skn-1(zu67) PUNIC/DMSO	19.0 ± 1.0	18.19 ± 0.1	n.s. ^f	3	295
skn-1(LG357) DMSO	27.0 ± 0.0	24.56 ± 0.2		3	218
skn-1(LG357) PUNIC/DMSO	28.3 ± 0.7	25.50 ± 0.4	< 0.01 ^g	3	194
daf-16(mu86) DMSO	23.0 ± 0.0	20.92 ± 0.3		3	346
daf-16(mu86) PUNIC/DMSO	23.0 ± 0.0	21.47 ± 0.2	< 0.01 ^h	3	327
hsf-1(sy441) DMSO	21.0 ± 0.0	19.82 ± 0.2		3	298
hsf-1(sy441) PUNIC/DMSO	20.3 ± 0.7	19.34 ± 0.2	n.s. ⁱ	3	345
aip-1(ok537) DMSO	23.7 ± 0.7	21.98 ± 0.4		3	287
aip-1(ok537) PUNIC/DMSO	22.3 ± 0.7	21.34 ± 0.1	n.s. ^j	3	278
xbp-1(zc12) DMSO	26.0 ± 0.0	23.62 ± 0.1		3	147
xbp-1(zc12) PUNIC/DMSO	26.0 ± 0.0	23.14 ± 0.3	n.s. ^k	3	160
hsp-4(VC1099) DMSO	22.0 ± 0.0	20.61 ± 0.1		3	252
hsp-4(VC1099) PUNIC/DMSO	24.0 ± 0.0	20.93 ± 0.1	n.s. ^l	3	286
atf-6(ok551) DMSO	31.0 ± 0.0	28.41 ± 0.3		3	283
atf-6(ok551) PUNIC/DMSO	31.0 ± 0.0	27.84 ± 0.1	n.s. ^m	3	317
ire-1(ok799) DMSO	22.0 ± 0.0	20.92 ± 0.1		3	238
ire-1(ok799) PUNIC/DMSO	22.0 ± 0.0	20.40 ± 0.2	< 0.05 ⁿ	3	236
pek-1(ok275) DMSO	25.7 ± 0.7	23.92 ± 0.3		3	344
pek-1(ok275) PUNIC/DMSO	26.3 ± 0.7	24.14 ± 0.1	n.s. ^o	3	356

¹ 75% quantile

Controls: ^a N2 DMSO, ^b N2 H₂O, ^c aak-2(ok524) DMSO, ^d pmk-1(km25) DMSO, ^e skn-1(zu135) DMSO, ^f skn-1(zu67) DMSO, ^g skn-1(LG357) DMSO, ^h daf-16(mu86), ⁱ hsf-1(sy441) DMSO, ^j aip-1(ok537) DMSO, ^k xbp-1(zc12) DMSO, ^l hsp-4(VC1099) DMSO, ^m atf-6(ok551) DMSO, ⁿ ire-1(ok799) DMSO, ^o pek-1(ok275) DMSO

Table 3: Results and statistical analyses of lifespan assays modulated by RO 90-7501

Strain, Substance, Solvent	Maximum Life Span [d] [+/- SEM] ⁱ	Mean Life Span [d] [+/- SEM]	P-value (vs. control, see footnotes)	Number of experiments [n]	Number of nematodes [n]
N2 DMSO	27.7 ± 0.7	24.66 ± 0.3		3	191
N2 RO/DMSO	29.0 ± 0.0	26.18 ± 0.2	< 0.001 ^a	3	210
N2 DMSO	24.0 ± 0.0	22.29 ± 0.1		3	308
N2 BHA/DMSO	24.0 ± 0.0	22.60 ± 0.2	n.s. ^a	3	339
N2 RO/BHA/DMSO	24.0 ± 0.0	22.71 ± 0.2	n.s. ^a	3	305
N2 RO/DMSO	26.0 ± 0.0	23.47 ± 0.4	< 0.001 ^a	3	305
aak-2(ok524) DMSO	20.7 ± 0.7	20.27 ± 0.3		3	191
aak-2(ok524) RO/DMSO	20.7 ± 0.7	20.10 ± 0.2	n.s. ^b	3	209
pmk-1(km25) DMSO	24.3 ± 0.0	21.82 ± 0.0		3	64
pmk-1(km25) RO/DMSO	25.0 ± 0.0	21.97 ± 0.0	n.s. ^c	3	57
skn-1(zu135) DMSO	25.0 ± 0.0	23.01 ± 0.2		3	284
skn-1(zu135) RO/DMSO	24.3 ± 0.7	22.59 ± 0.3	n.s. ^d	3	237
skn-1(zu67) DMSO	23.0 ± 0.0	22.20 ± 0.1		3	185
skn-1(zu67) RO/DMSO	24.0 ± 1.0	22.50 ± 0.0	n.s. ^e	3	183
skn-1(LG357) DMSO	22.0 ± 0.0	21.05 ± 0.2		3	175
skn-1(LG357) RO/DMSO	23.3 ± 0.7	21.22 ± 0.2	n.s. ^f	3	181
daf-16(mu86) DMSO	24.3 ± 0.7	22.38 ± 0.1		3	368
daf-16(mu86) RO/DMSO	23.7 ± 0.7	22.13 ± 0.2	n.s. ^g	3	316
hsf-1(sy441) DMSO	22.0 ± 1.0	20.52 ± 0.7		3	298
hsf-1(sy441) RO/DMSO	21.0 ± 0.0	19.81 ± 0.3	n.s. ^h	3	343
aip-1(ok537) DMSO	23.7 ± 0.7	21.98 ± 0.4		3	287
aip-1(ok537) RO/DMSO	22.3 ± 0.7	21.52 ± 0.5	n.s. ⁱ	3	304
xbp-1(zc12) DMSO	26.0 ± 0.0	23.62 ± 0.1		3	147
xbp-1(zc12) RO/DMSO	25.3 ± 0.7	22.89 ± 0.4	n.s. ^j	3	140
hsp-4(VC1089) DMSO	22.0 ± 0.0	20.61 ± 0.1		3	252
hsp-4(VC1089) RO/DMSO	22.0 ± 0.0	20.40 ± 0.1	n.s. ^k	3	273
atf-5(ok576) DMSO	24.5 ± 1.2	22.84 ± 0.4		3	120
atf-5(ok576) RO/DMSO	23.0 ± 0.0	22.51 ± 0.4	n.s. ^l	3	125
atf-6(ok551) DMSO	31.0 ± 0.0	28.41 ± 0.3		3	283
atf-6(ok551) RO/DMSO	33.0 ± 0.0	29.17 ± 0.2	< 0.05 ^m	3	293
ire-1(ok799) DMSO	22.0 ± 0.0	20.92 ± 0.1		3	238
ire-1(ok799) RO/DMSO	23.0 ± 1.0	20.84 ± 0.3	n.s. ⁿ	3	212
pek-1(ok275) DMSO	25.7 ± 0.7	23.92 ± 0.3		3	344
pek-1(ok275) RO/DMSO	25.0 ± 0.0	23.37 ± 0.2	< 0.05 ^o	3	235

ⁱ 75% quantile

Controls: ^a N₂ DMSO, ^b aak-2(ok524) DMSO, ^c pmk-1(km25) DMSO, ^d skn-1(zu135) DMSO, ^e skn-1(zu67) DMSO, ^f skn-1(LG357) DMSO, ^g daf-16(mu86), ^h hsf-1(sy441) DMSO, ⁱ aip-1(ok537) DMSO, ^j xbp-1(zc12) DMSO, ^k hsp-4(VC1089) DMSO, ^l atf-5(ok576) DMSO, ^m atf-6(ok551) DMSO, ⁿ ire-1(ok799) DMSO, ^o pek-1(ok275) DMSO

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Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel „Promoting Stress Resistance and Lifespan by Modulators of Mitochondrial Metabolism“ selbstständig angefertigt habe.

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